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2. ***Tables, Foot-Notes, Figure Legends, etc.*** Tables should be typed on separate sheets and placed after the Literature Cited. Because of the high cost of setting such material in type authors are earnestly requested to limit tabular material as much as possible. Similarly, foot-notes to tables should be avoided wherever possible. If they are essential, they should be indicated by asterisks, daggers, etc., rather than by numbers. Foot-notes are not normally permitted in the body of the text. Such material should be incorporated into the text where appropriate. Explanations of figures should be typed double-spaced and placed on separate sheets at the end of the paper.

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THE EFFECTS OF AN ISOPOD CASTRATOR, *PROBOPYRUS PANDALICOLA*, ON THE SEX CHARACTERS OF ONE OF ITS CARIDEAN SHRIMP HOSTS, *PALAEMONETES PALUDOSUS*

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Parasitic castration is the "destruction or alteration of gonad tissue, reproductive behavior, hormonal balance or other modification of host reproductive effort above and beyond that which results from a nonselective use of host energy reserves by the parasite" (Baudoin, 1975). Baudoin viewed parasitic castration as a parasite's adaptation and hypothesized that, as a result of the curtailment of host reproduction, the fitness of the parasite is increased due to increased host survivorship, increased host growth and/or increased energy available to the parasite.

Parasitic castration of Crustacea was reviewed by Reinhard (1956), who discussed the external and internal host modifications and also the theories attempting to explain crustacean castration caused by rhizocephalan barnacles and epicaridean isopods. Parasitic castration of crustacean hosts has been suggested as being due to: nutritional drain by the parasite (Reinhard, 1956); reduction in the titers of circulating reproductive hormones as a consequence of large blood losses to the parasite (Walker, 1977); secretion of toxic substances (Reinhard, 1956); indirect hormonal castration (Baudoin, 1975); or, in male hosts, decline or cessation of secretion by the androgenic glands (Charniaux-Cotton, 1960). The parasitic castrator either retards the development of the gonads or actually destroys the sex cells, resulting in a complete atrophy of the gonad (Reinhard, 1956).

Epicaridean isopods generally cause a lesser reduction of the gonads of the male than the female host, whereas the external secondary sex characters of the male host are modified more frequently and to a greater degree than those of the female (Reinhard, 1956). The effect of the castrator on the external sex characters of the male host is usually referred to as a feminization or a juvenilization, as these characters are reduced in size and/or resemble those of the female host. In the bopyrid epicarideans, which infect decapod crustaceans, the effects on the host sex characters are varied. In fact, several workers have reported a lack of effect of bopyrid infection on host testes (Reverberi, 1941; Danforth, 1963; Codreanu, Codreanu, and Pike, 1965; Pérez, 1923; Wenner, 1978; Reinhard, Von Brand,

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and McDuffie, 1947), on host ovaries (Danforth, 1963; Pérez, 1923; Codreanu *et al.*, 1965; Bourdon, 1968; Reinhard *et al.*, 1947), and on external sex characters of both host sexes (Callan, 1940; Pérez, 1923; Delye, 1955; Reinhard *et al.*, 1947; Codreanu *et al.*, 1965; Bourdon, 1968; Nöel, 1976).

Probopyrus pandalicola (Packard) (= *P. floridensis* Richardson) is a branchial bopyrid ectoparasitic on 16 species of palaemonid shrimps, that range from New Hampshire, U. S. A., to Sao Paulo, Brazil (see Beck, 1979). The effects of *P. pandalicola* on the sex characters of the marine shrimp *Palaemonetes vulgaris* (Say) were studied by Morris (1948); however, the study was incomplete, and a statistical treatment of the data was lacking. Recent studies of *P. pandalicola* on an estuarine host *Palaemonetes pugio* Holthuis, provided information on energetic relationships (Anderson, 1977) and on hematophagy (Walker, 1977).

The present study examined the effects of *P. pandalicola* on the sex characters of its freshwater host *Palaemonetes paludosus* (Gibbes). These results are compared with other published information on the effects of bopyrid infection, and the mechanisms of parasitic castration are discussed in the light of new information on reproductive endocrinology of decapod Crustacea (Charniaux-Cotton, 1975a, b, 1976; Payen, 1973, 1974; Touir, 1977a, b).

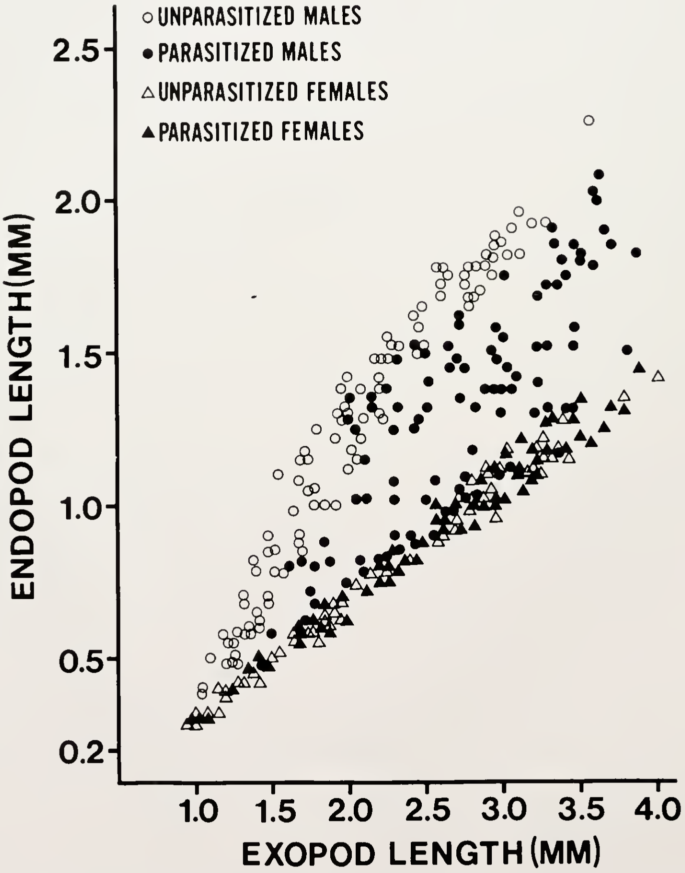

FIGURE 1. Relationship of endopod length to exopod length of the first pleopod of parasitized and unparasitized specimens of *P. paludosus*.

TABLE I

Statistics concerning regressions of endopod length (Y) on exopod length (X) for parasitized and unparasitized male and female specimens of P. paludosus.

	Unparasitized males	Parasitized males	Unparasitized females	Parasitized females
Regression Y =	$1.492 \log_e X + 0.222$	$0.504 X - 0.071$	$0.386 X - 0.084$	$0.383 X - 0.076$
r	0.981	0.811	0.992	0.986
N	100	100	60	60
s.e. of slope	0.030	0.037	0.007	0.009
s.e. of Y-intercept	0.022	0.102	0.016	0.023

MATERIALS AND METHODS

Infected and uninfected specimens of *Palaemonetes paludosus* were collected from McBride's Slough (at State Highway, 267), a spring-fed tributary of the Wakulla River, Florida. Total lengths of the external sex characters (viz., endopod of first pleopod, appendix masculina and chela) were measured to the nearest 0.01 mm with a calibrated ocular micrometer, and these lengths were regressed (least squares analysis) and plotted with total body length, exopod length of first pleopod, or endopod length of second pleopod. The lengths of the latter two structures were highly correlated with total body length, and there were no differences in length of these structures between the same size parasitized and nonparasitized shrimp of different sexes. Hosts with recently acquired parasites were not included in the analysis, as the host must molt before the effects on the external sex characters are evident. The gonads were excised, and the total length was measured to the nearest 0.01 mm with a calibrated ocular micrometer. Gonads and gonoducts were embedded in paraffin, sectioned at 10 μm thickness and stained with Harris' hematoxylin and alcoholic eosin. Oocytes were classified as mature if yolk granules were present, and immature if yolk granules were absent. The diameter of the largest immature and mature oocytes from each sectioned ovary was measured to the nearest 0.01 mm.

RESULTS

Secondary sex characters

There was little sexual difference in endopod length of the first pleopod of small, unparasitized shrimp, but as shrimp increased in size, the difference was pronounced (Fig. 1). The relationship between endopod and exopod length was linear in unparasitized females but logarithmic in unparasitized males (Fig. 1 and Table I), indicating that in unparasitized males the growth rate of the endopod relative to that of the exopod was faster in smaller than in larger individuals. Parasitism had no effect on the endopod length in females, since there were no significant differences between parasitized and unparasitized females in the slopes and elevations of the regression equations relating endopod to exopod length (analysis of covariance, $P > 0.2$). However, parasitism had a marked effect on the endopod length of male hosts. Most parasitized males had endopod lengths intermediate between those of unparasitized males and females, but some males showed no effects of parasitism on endopod length (see Fig. 1: closed circles within or adjacent to the cluster of open circles). Other males had endopods as small as those of females at the same exopod length (closed circles within or adjacent to

TABLE II

Statistics concerning regressions of appendix masculina length (Y) on endopod length (X) for parasitized and unparasitized male specimens of P. paludosus.

	Unparasitized males	Parasitized males	Recalculation	
			Unparasitized males	Parasitized males
Regression Y =	$1.143 \log_e X + 0.263$	$1.164 \log_e X + 0.119$	$1.1535 \log_e X + 0.256$	$1.1535 \log_e X + 0.128$
r	0.983	0.940		
N	80	80		
s.e. of slope	0.026	0.026		
s.e. of Y-intercept	0.019	0.046		

clusters of triangles). The relatively low correlation of endopod with exopod length in parasitized males (Table I) was due to the large variation in the effects of parasitism on endopod length.

The relationship of the size of the appendix masculina to the length of the endopod of the second pleopod was logarithmic for both male groups, indicating that the growth rate of the appendix masculina relative to that of the endopod was

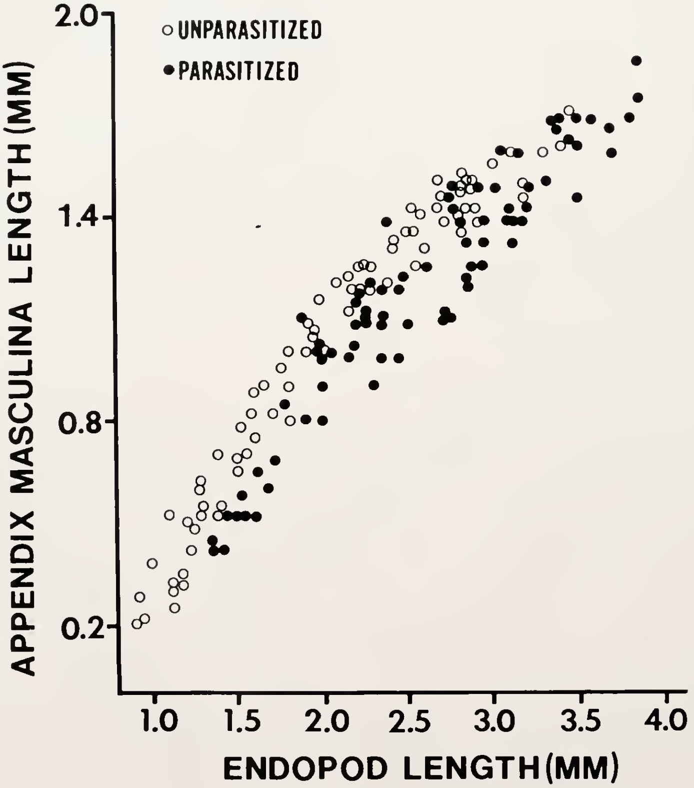

FIGURE 2. Relationship of appendix masculina length to exopod length of the second pleopod of parasitized and unparasitized male specimens of *P. paludosus*.

faster in smaller than in larger males. The data were best fitted with a regression line involving a log transformation of the X variable (endopod) (Table II). The appendix masculina was, on the average, shorter in parasitized males, although its length in many males was unaffected by parasitism (see Fig. 2; closed circles within the cluster of open circles). Since the analysis of covariance indicated that there were significant differences between parasitized and unparasitized shrimp in elevations ($P < 0.01$) but not in slopes ($P > 0.2$) of the regression equations relating size of the appendix masculina to endopod length, the average difference in length of the appendix masculina between the male groups at any endopod length can be calculated. However, the Y-intercept must be recalculated for each equation by using the joint mean of the slopes (Table II); otherwise, large errors might result if the original Y-intercepts are used, because each was determined from a different slope, even though the difference is small (see Gould, 1971). The ratio of the antilogs (largest on top) of the recalculated Y-intercepts resulted in the average length of the appendix masculina of unparasitized males being 1.14 times greater than that of parasitized males at any endopod length.

Chela length was strongly correlated with total length of female and male shrimp (Figs. 3 and 4, Table III). There were no significant differences between parasitized and unparasitized female hosts in slopes or elevations of the regression equations relating chela to total body length (analysis of covariance, $P > 0.2$), whereas significant differences occurred in the slopes ($P < 0.01$) between parasitized and unparasitized male hosts. Furthermore, slopes and elevations were not significantly different between the regression equations for parasitized males and both female groups ($P > 0.2$), but were different between the regression equations for unparasitized males and both female host groups ($P < 0.01$).

Unparasitized female shrimp developed breeding setae on the ventral side of the thorax and abdomen during the ovigerous intermolt, but these setae were ab-

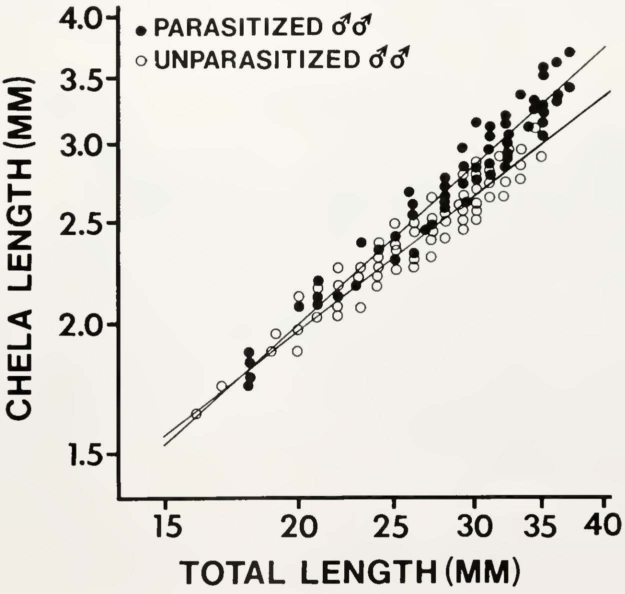

FIGURE 3. Log-log relationship of chela length to total length in parasitized and unparasitized male specimens of *P. paludosus*.

TABLE III

Statistics concerning regressions of chela length (Y) on total body length (X) for parasitized and unparasitized male and female specimens of P. paludosus.

	Unparasitized males	Parasitized males	Unparasitized females	Parasitized females
Regression $\log_e Y$ =	$0.783 \log_e X - 1.657$	$0.913 \log_e X - 2.043$	$0.914 \log_e X - 1.990$	$0.946 \log_e X - 2.114$
r	0.973	0.973	0.982	0.985
N	60	60	60	60
s.e. of slope	0.024	0.028	0.016	0.015
s.e. of Y-intercept	0.078	0.096	0.053	0.050

sent from parasitized females. These additional setae function in egg bearing (Antheunisse, Van Den Hoven, and Jefferies, 1968).

Ovary

The gonads of 13 unparasitized and 12 parasitized female shrimp, ranging from 15 to 41 mm and 22 to 41 mm long, respectively, were examined. Except for two unparasitized shrimp, all were collected during the breeding season.

Ovarian size in unparasitized females was dependent on total body length and stage of ovarian activity. For example, the ovary of a 41-mm-long uninfected female, immediately prior to oviposition, was 8 mm long and extended from a point anterior to the stomach, posteriorly into the anterior abdomen, whereas a 40-mm-long uninfected ovigerous female with a recently deposited brood possessed a 3.5-mm-long ovary that extended only to the posterior margin of the thorax. The ovaries of the largest parasitized females were less than 3.3 mm long.

Ovaries of uninfected mature females (see Fig. 5) simultaneously contained immature oocytes (devoid of yolk) and mature oocytes (480–900 μm in diameter) with varying deposits of yolk granules, depending on the state of ovarian activity. Unparasitized mature females collected out of the breeding season, immature

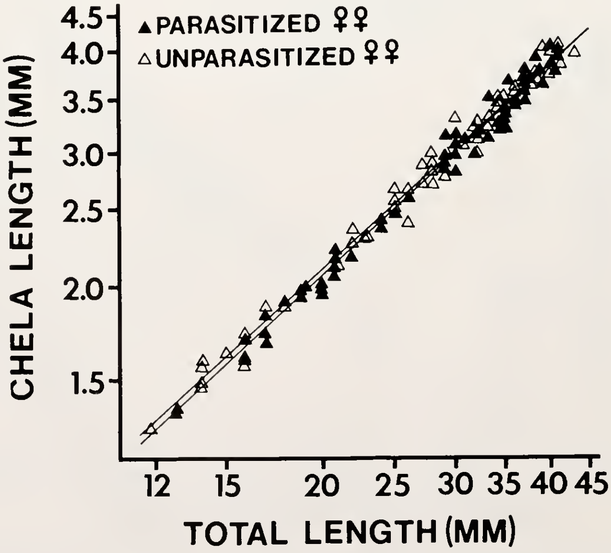

FIGURE 4. Log-log relationship of chela length to total length in parasitized and unparasitized female specimens of *P. paludosus*.

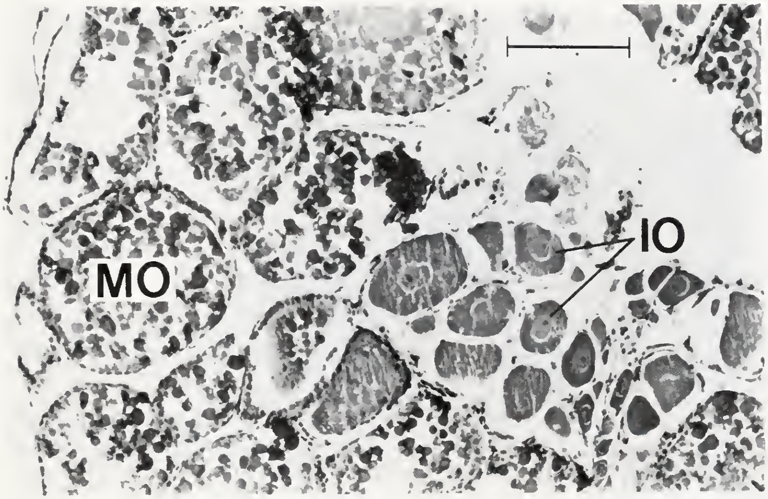

Figure 5. Cross section through the ovary of an unparasitized, mature female specimen of *P. paludosus* with mature (MO) and immature oocytes (IO). Note the many yolk granules in the mature oocytes. Scale = 500 μm.

shrimp and parasitized shrimp (Fig. 6) had ovaries containing only immature oocytes. No yolk granules were ever observed in the oocytes of parasitized females.

Out of 303 infected mature (>27 mm long, see Beck, 1979) female shrimp collected, only five were ovigerous, and two others had ripe ovaries (seen through the carapace). The infections of all these female shrimp were relatively recent, since two ovigerous shrimp carried only cryptonisci (isopod larvae), two carried young female isopods less than 1.2 mm long and one carried a 2.1 mm female isopod; one of the nonovigerous shrimp with ripe ovaries carried a cryptoniscus, and

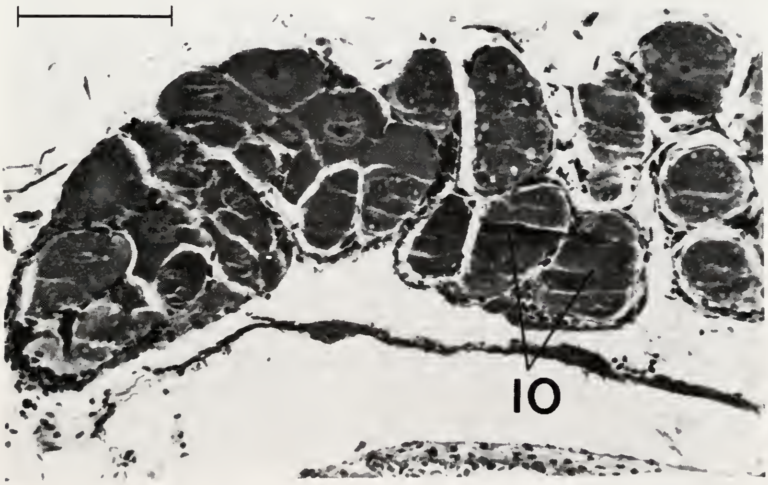

Figure 6. Longitudinal section through the ovary of a parasitized 35-mm-long female specimen of *P. paludosus* with only immature oocytes (IO). Scale = 250 μm.

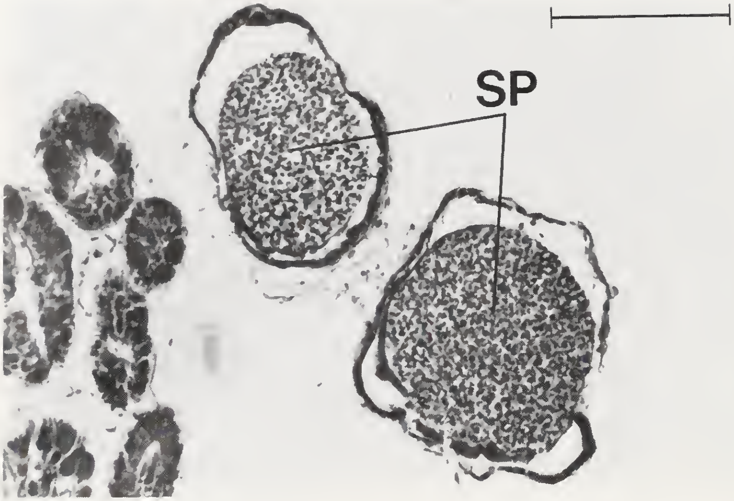

FIGURE 7. Cross section through the loop of a vas deferens of a parasitized male specimen of *P. paludosus* with densely packed spermatozoa (SP). Scale = 250 μm.

the other carried a 1.5-mm-long female parasite. Four of the ovigerous shrimp had recently deposited broods, and the other carried a mature brood.

Testis

The gonads of 14 uninfected and 17 infected male shrimp, ranging from 16 to to 35 mm and 18 to 43 mm long, respectively, were examined. Parasitism appeared to have no effect on the size of the testes, as testes of unparasitized and parasitized shrimp of the same body length were similar in size. The total testicular lengths of unparasitized and parasitized shrimp ranged from 2.0 to 4.1 mm and 2.2 to 4.1 mm, respectively, depending on total length of the specimen. Furthermore, spermatozoa were numerous and in dense masses in the vas deferens and testes of parasitized shrimp (Fig. 7), and no difference could be observed from unparasitized males. Spermatozoa occurred in the vas deferens of males as small as 19 mm long, although densely packed spermatozoa were not present below 21 mm long. Abundant spermatozoa occurred in the vas deferens of shrimp during all seasons. Spermatophores were seen protruding from the genital openings of 12.2% (N = 458) of the unparasitized and 11.1% (N = 190) of the parasitized shrimp, ranging from 23 to 37 mm and 21 to 37 mm in total length, respectively.

DISCUSSION

The modification of the secondary sex characters of male but not female specimens of *Palaemonetes paludosus* by *Probopyrus pandalicola* supports the general rule of Reinhard (1956) that epicarideans affect the secondary sex features of the male host more frequently and to a greater degree than the females. The average length of the endopod of the first pleopod and that of the appendix masculina in infected male *P. paludosus* was reduced in size, whereas that of the chela was larger than in nonparasitized males. Some parasitized males showed a considerable size reduction in these structures, but there was no size reduction in other males.

TABLE IV

Reanalysis of Morris' (1948) data: statistics concerning regressions of chela length (Y) on carapace length (X) for unparasitized and parasitized male and female specimens of P. vulgaris.

	Unparasitized males	Parasitized males	Unparasitized females	Parasitized females
Regression $\log_e Y$ =	$0.867 \log_e X - 1.137$	$0.763 \log_e X - 0.819$	$1.210 \log_e X - 1.883$	$1.277 \log_e X - 2.123$
r	0.698	0.891	0.924	0.934
N	31	11	92	38
s.e. of slope	0.165	0.130	0.053	0.081
s.e. of Y-intercept	0.396	0.320	0.141	0.208

The result is that most parasitized males are externally more similar to female hosts than to uninfected males. This parasitic effect is not entirely a juvenilization or size reduction of the secondary sex characters, as interpreted by Baudoin (1975), but is also a feminization, since chela growth of parasitized males is greater than that of nonparasitized males.

Morris (1948) reported that in *Palaemonetes vulgaris* parasitized by *Probopyrus pandalicola* there was an "apparent tendency" for the growth of the male chela relative to carapace length to approach that of normal females, whereas the relative growth of female chela approached that of normal males. However, my statistical reanalyses (using least squares regressions and log-log transformations) of Morris' data revealed no significant differences in the slopes and y-intercepts of the regression equations describing the chela-to-carapace-length relationship between parasitized and unparasitized males ($P > 0.5$), or between parasitized and unparasitized females ($P > 0.3$) (Table IV). These findings indicate that parasitism did not affect the relative growth of the chela in either sex. Thus, in contrast to the effects of *P. pandalicola* on *P. paludosus* reported herein, *P. pandalicola* did not appear to affect male chela growth in *P. vulgaris.* In addition, Morris (1948) suggested that the appendix masculina (of only six shrimp) was not "materially affected" by parasitism. Furthermore, Callan (1940) concluded that *Bopyrus squillarum* had no effect on the endopod of the first pleopod or on the appendix masculina of three species of *Palaemon* (= *Leander*).

Modification of the permanent secondary sex characters of female hosts by bopyrids is rare. Tucker (1930) found a slight reduction in growth of the chela and first pleopod of female *Upogebia pusilla* (Petagna) (= *U. littoralis*) parasitized by *Gyge branchialis.* Bourdon (1968) reported a diminution of the length of the three last pleopods of female specimens of *Munida perarmata* A. Milne Edwards and Bouvier parasitized by *Pseudione crenulata* Sars.

Temporary breeding characters are absent from many female hosts parasitized by bopyrids (*e.g.*, Callan, 1940; Yoshida, 1952; Nöel, 1976; present study), but these characters are rarely examined even when external female host modifications are studied. Yoshida (1952) and Nöel (1976) found that the breeding dress of the caridean shrimps *Palaemon serrifer* and *Processa edulis*, respectively, were continually present when abdominally parasitized but were either absent or poorly developed when branchially parasitized. Furthermore, Allen (1966) reported that another caridean shrimp *Pandalus montagui*, abdominally parasitized by *Hemiarthrus abdominalis,* precociously developed the breeding dress, but that not all parasitized females developed these breeding characters. Allen suggested that the enlarged coxal plates of the breeding dress might offer added protection to the parasite in its location beneath the anterior part of the host abdomen. Hartnoll (1967) found that rhizocephalan barnacles caused their crab host to resemble a mature ovigerous female both physically and behaviorly. The externa of the barnacle occupies the abdominal position and plays the role of the host egg mass. The continual breeding dress provides adequate room and protection for the barnacle, and the behavioral modifications cause the crab to clean and ventilate the parasite as if it were an egg mass. Likewise, abdominal bopyrids, which usually lie beneath the sterna of the first three or four segments (Bruce, 1973) where the eggs of infected shrimp are deposited, might receive the same benefits since they maintain the host breeding dress in contrast to branchial bopyrids. Caridean shrimp in breeding dress, which normally is present only during the intermolt of egg deposition, have a wider sternum (Yonge, 1955; Antheunisse *et al.*, 1968), larger abdominal pleura (Höglund, 1943; but not Antheunisse *et al.*, 1968), additional setae (Antheunisse *et al.*, 1968) and the presence of white chromatophores on the epimera (Antheunisse *et al.*, 1968). The first two above breeding characters would provide additional room and more protection to the parasite, and, as Antheunisse *et al.* (1968) suggested for ovigerous females, the white chromatophores might partially camouflage the otherwise transparent shrimp which is probably more visible to a predator because of the presence of the parasite (or egg mass on uninfected shrimp). In addition, ovigerous caridean shrimp clean their eggs with their chela and ventilate them with a back-and-forth movement of the pleopods (Bauer, 1976; Law, 1947; Burkenroad, 1947; Ewald, 1969; Ruello, Moffit, and Phillips, 1973; Schone, 1961; Phillips, 1971); and, if females are maintained in breeding dress, the same "service" might be given to the abdominal bopyrid. Thus abdominal bopyrids, like rhizocephalan barnacles, might allow the female host to remain in breeding dress to obtain the benefits afforded the host egg mass.

The effects of *Probopyrus pandalicola* on the gonads of *P. paludosus* support the general rule of Reinhard (1956) that epicaridean infection causes suppression of the gonads of the female host and a lesser reduction of the male host gonads, although in the present study there was no effect on the male host gonads. In contrast, Morris (1948) found that infection of male *Palaemonetes vulgaris* by *P. pandalicola* reduced the number of sperm in the host testes, although there was no appreciable difference in testicular size between parasitized and unparasitized male shrimp. Likewise, in another palaemonid shrimp, Callan (1940) reported that infection of *Palaemon* spp. by *Bopyrus squillarum* decreased the rate of sperm production. Among male host-bopyrid associations, only Delye (1955) found almost total testicular atrophy to be the typical parasite effect. Variable effects of a bopyrid on the same host species, ranging from slight reduction to complete atrophy of the testes, were reported by Tucker (1930), Menon (1953) and Hiraiwa and Sato (1939).

The effect of bopyrids on the ovaries of female palaemonid hosts has been nearly identical: a retardation of development (Callan, 1940; Morris, 1948; Yoshida, 1952; present study). Maximum ovarian size of parasitized hosts is considerably less than those of uninfected females. Yolk is never stored in the oocytes, which remain in an immature state very much like those of immature females or mature females out of the breeding season. The ovaries of most other female caridean shrimp are similarly affected by bopyrids (Pike, 1961; Allen, 1966; Reverberi, 1952; Bourdon, 1968; Wenner, 1978); however, Danforth (1963, p. 63) found that, of 214 ovigerous *Crangon* (= *Crago*) *nigromaculata* Lockington parasitized by *Argeia pugettensis* Dana, "most had a normal or nearly normal clutch." As in the present paper, a small number of infected ovigerous hosts were reported in host-parasite associations where castration is the usual parasite effect (Williams and Brown, 1972; Bruce, 1968; Truesdale and Mermilliod, 1977; Callan, 1940; Kruczynski and Menzies, 1977; Pike, 1960; Allen, 1966; Bourdon, 1968). The ability of these infected female hosts to reproduce might in some cases be due to the mature host's acquisition of young parasites, with host egg production occurring before the parasite can exert its castration effect.

Baudoin (1975) stated that the effects of parasitic castration would depend on host sex, since host reproductive effort will take different forms in each sex. In the Crustacea, female host gonads are generally more severely affected than are those of male hosts, since ovaries, with their large amount of lipids stored in mature oocytes, are more expensive to maintain than testes (Veillet, 1945; Baudoin, 1975). In addition, a castrated female host would have no brood to maintain, would be less vulnerable to predation and could devote more energy to the parasite. (This would not apply to penaeidean shrimp hosts, since their eggs are released directly into the water and lack parental care.)

Male crustaceans have their secondary sex characters more frequently and more severely affected by parasitic castrators than do female hosts, since they can expend much energy in competing for and seducing females. Findings from the present study did not indicate whether mating behavior was inhibited by infection. The parasite could achieve inhibition of mating behavior by making an infected female host less "desirable" to the male or by reducing the mating drive in the male. Male caridean shrimp apparently sense, through a non-diffusible substance on the exoskeleton of a female that has recently molted into the breeding dress, the readiness of the female to copulate (Bauer, 1976). However, this non-diffusible substance might never be produced, since the breeding dress does not appear in branchially infected females (see above). Moreover, Hartnoll (1967) reported that many female hosts infected with rhizocephalan barnacles failed to copulate.

Numerous explanations have been offered for the means by which crustacean castrators produce the effects on the primary and secondary sex characters of the host (see Reinhard, 1956; Baudoin, 1975; Hartnoll, 1967), but the most recent views suggest some combination of copious nutrient withdrawal by these relatively large parasites and interference with the host endocrine system (Hartnoll, 1967; Baudoin, 1975; Walker, 1977). The simplest explanation for female hosts would be that the loss of large amounts of nutrients (up to 25% of the total volume of the host hemolymph daily, see Walker, 1977) does not allow any energy for the maintenance of the expensive ovary but only for that of somatic tissues. Nutritional stress as a cause of crustacean castration has also been suggested by Veillet (1945) and Field (1969). Calow (1973) reported that undernourishment prevented maturation of the follicles, caused atrophy of follicles, or resulted in the resorption of ova in iteroparous species.

Another possible explanation of host castration could be that the parasite either causes continual secretion of a gonad-inhibiting hormone (GIH) secreted by the host's ganglionic X-organ and sinus gland complex, or that the parasite secretes this hormone. GIH inhibits ovarian growth and vitellogenesis (Adiyodi and Adiyodi, 1970; Charniaux-Cotton, 1975a), and, through such influences as photoperiod, GIH can regulate the seasonality of ovarian growth in many decapods. Thus, the similarities between the ovarian condition of mature females not in the breeding season and that of parasitized females reported herein might not be surprising. Suppression of ovarian development by crustacean castrators is not permanent. If the parasite dies before the host, or is detached from the host, regeneration or maturation of the ovary occurs (Hiraiwa and Sato, 1939; Delye, 1955;

Pike, 1960; Kuris, 1971; Wenner, 1978; present paper) and broods can subsequently be produced (Pike, 1960; Kuris, 1971, 1974).

In female decapods, the development of the permanent and temporary sex characters appears to be directly controlled by the ovaries (Charniaux-Cotton, 1975a, b) and indirectly by GIH. However, in females parasitized by abdominal bopyrids or rhizocephalan barnacles, the temporary sex characters are present even though ovarian development is arrested (see above), whereas when female hosts are branchially parasitized, these characters are absent. Hartnoll (1967) hypothesized that rhizocephalans must, by some means, produce or cause to be produced the hormones normally manufactured by the ovary. In addition, it seems likely that nutritional stress could prevent the appearance of the temporary sex characters since ovarian development is inhibited; this might explain the loss of breeding dress in hosts infected with branchial bopyrids, but not the presence of these characters in hosts infected with abdominal bopyrids or rhizocephalans.

Mechanisms of parasitic castration in male hosts appear to be more complex due to the variable nature of the effects on the testes and the presence of androgenic glands (gonadal hormones are lacking). These glands produce male hormones and contribute to spermatogenesis and the development of the secondary sex characters during and after puberty (Charniaux-Cotton, 1976). However, in gonochoristic shrimp, androgenic hormone is not needed for the maintenance of the external sex characters, once formed (Touir, 1977b). In addition, there have been recent discoveries of local inducers of male sex differentiation even before the androgenic glands have developed (Payen, 1973, 1974; Charniaux-Cotton, 1975a), and the presence of possibly two male hormones secreted by the median protocerebrum, which are responsible for the maintenance of the testes, sperm ducts and androgenic glands (Touir, 1977a).

Since smaller amounts of energy are required to maintain the testes (Veillet, 1945), it would appear to be more difficult for parasitic castrators to nutritionally stress testes than ovaries. Thus, only a reduction in sperm production usually occurs in infected males, in contrast to total maturational arrest of ovaries of infected females. An alternative explanation for male host castration is that the androgenic glands are destroyed by the parasite (see Veillet and Graf, 1958). Touir (1971b) found that termination of androgenic hormone production in shrimp reduced the intensity of spermatogenic activity. In the uncommon cases of complete testicular atrophy in hosts (see Delye, 1955), parasitic interruption of the production of the protocerebral hormone might cause the testes to degenerate. Touir (1977a) reported that cessation of production of this hormone in protandric shrimp caused testes degeneration before the change to the female life phase.

As in female hosts, the removal or loss of the parasite from male hosts allows the gonads to regenerate and return to active spermatogenesis (Hiraiwa and Sato, 1939; Delye, 1955; Callan, 1940; Pike, 1960). However, the feminized or retarded male external sex characters do not return to the normal male size or shape (Tucker, 1930; Veillet and Graf, 1958). Veillet and Graf (1958) found that the extent of the degeneration of the androgenic gland was correlated with the degree of the modification of the external male characters in anomuran and brachyuran crabs, and that when the gland atrophies, it is incapable of regeneration, unlike the testes. However, Touir (1977b) reported that androgenic hormones were not responsible for the maintenance of the external sex characters in gonochoristic shrimp. Furthermore, the external sex characters of male hosts can be affected, but not the testes of hosts infected by bopyrids (Reverberi, 1941;

present paper) and some Rhizocephala (Reinhard, 1956). On the other hand, the testes are reduced or atrophied but the external sex characters are unaffected in some male hosts infected with bopyrids (Callan, 1940; Delye, 1955) and in many infected with entoniscids (Kuris, 1971). The absence of parasitic effects on both primary and secondary sex characters of infected hosts is known for several host-castrator relationships.

As Nöel (1976) stated, the mode of action of parasitic castrators on the sexuality of decapods remains very confused in view of the diversity of host reactions, the interventions of the specific nature of the parasite and the incomplete knowledge of decapod reproductive endocrinology. Further experimental and descriptive research is needed in these areas with emphasis on detailed morphometric studies of the permanent and temporary secondary sex characters of the host; the endocrinological nature of the host median protocerebrum, ovaries, androgenic glands, and the X-organ and sinus gland complex; and the determination of the presence some combination of a nutritional drain and/or a hormonal interference by the parasite.

SUMMARY

Effects of the parasitic isopod, *Probopyrus pandalicola*, on the sex characters of its host shrimp, *Palaemonetes paludosus*, are described, and possible mechanisms for these effects are discussed. *P. pandalicola* sterilize the female host by preventing ovarian maturation but do not affect the external sex characters. In contrast, the growth of the external sex characters of the male host was either accelerated (chela) or retarded (pleopod structures), but there was no parasitic effect on the testes. The mechanism for these castration effects appears to be some combination of a nutritional drain and/or a hormonal interference by the parasite.

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SALT AND WATER BALANCE IN TWO MARINE SPIDER CRABS, *LIBINIA EMARGINATA* AND *PUGETTIA PRODUCTA*.

III. SOME FACTORS INVOLVED IN SHORT-TERM ADAPTATION TO A DILUTE MEDIUM

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Classical studies (Bethe, 1929; Schlieper, 1929; Bialaszewicz, 1931; Margaria, 1931; Hukuda, 1932; Schwabe, 1933; Nagel, 1934; Huf, 1936; Webb, 1940) have demonstrated that crabs from marine and brackish-water habitats are permeable to salts and water and produce urine at substantial rates. Thus, when an osmoconforming decapod is exposed to a dilute salinity, it is expected that the change in blood concentration will be accompanied by a temporary increase in volume, elimination of salts and water via the urine, and diffusion of salts across the body wall. All of these events are known to occur in osmoconforming crabs; however, there have been few studies in which it has been possible to evaluate and compare the relative importance of these factors. The present study is an attempt to evaluate those factors which are of importance in the short-term salinity adaptation of the osmoconforming spider crabs, *Pugettia producta* (Randall, 1839) and *Libinia emarginata* (Leach, 1815).

MATERIALS AND METHODS

Specimens of *Pugettia producta* were maintained at the University of California, Berkeley at 10 to 12° C in filtered sea water (SW) (about 1015 milliosmoles) obtained from the Bodega Marine Laboratory, Bodega Bay, California. Specimens of *Libinia emarginata* were maintained at the Marine Biological Laboratory, Woods Hole, Massachusetts at 19 to 21° C in running SW (about 945 milliosmoles).

Methods used in handling samples of blood and urine, and in the determinations of urine production rates, inorganic ion concentrations, and weight changes have been previously described (Cornell, 1979a). Measurements of hydrostatic pressure were made with a Statham model P23BC pressure transducer and the results displayed on a Grass Instruments Co. model 7a polygraph. The pressure transducer was connected via Intramedic P. E. 190 tubing to an 18 gauge hypodermic needle which was inserted through the arthrodial membrane of the first leg base.

Dilutions of SW were made with tap water and initially checked and/or adjusted with a Goldberg refractometer (American Optical Co.), and/or a Perkin-Elmer model 290 atomic absorption spectrophotometer at Berkeley, or a set of hydrometers at Woods Hole. Except for running SW, all media were continuously aerated.

RESULTS

Weight changes and urine production rates in Libinia *and* Pugettia

Weight changes in normal and nephropore-occluded specimens of *Libinia* and *Pugettia* transferred from 100 to 80% SW are presented in Figure 1. In normal crabs, maximum weight gains occurred during the first hour after transfer to 80% SW and weight returned to normal by about the tenth hour. *Pugettia* gained a maximum of 1.4% body weight (bw) compared with 0.56% bw for *Libinia*. Nephropore-occluded crabs of both species gained at least 5% bw after 4 hr of exposure to 80% SW.

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Empirically, it has been found that an equation of the form $y = Ct \exp(-kt)$ provides a good description of average weight changes during the first several hours of exposure to 80% SW. Data may be fitted to this equation by regressing

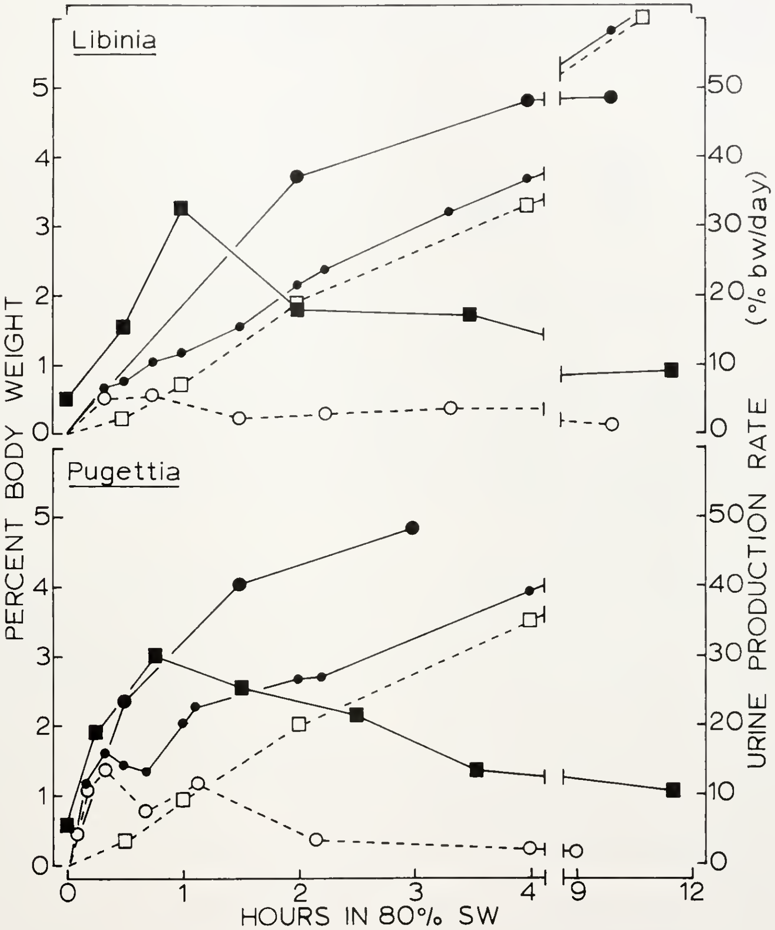

FIGURE 1. Water influx as urine production and volume change in specimens of *Libinia emarginata* and *Pugettia producta* transferred from 100 to 80% sea water. For both species, values for urine production rates (*Libinia*, N = 2; *Pugettia*, N = 9) are indicated by the solid squares and are read from the right ordinates. Values for the data which follow are read from the left ordinates. Open squares indicate the cumulative volume of urine produced from time 0 and are based on the above data. The larger solid circles indicate the weight gains in nephropore-occluded crabs (*Libinia*, N = 7; *Pugettia*, N = 7). Open circles indicate the weight gains in normal crabs (*Libinia*, N = 19; *Pugettia*, N = 10). The smaller solid circles indicate the calculated sum of the cumulative volume of urine, and the weight gain in normal crabs, an estimate of the total amount of water which entered the crab since time 0. Note that there is a discontinuity and a change of scale in the abscissa.

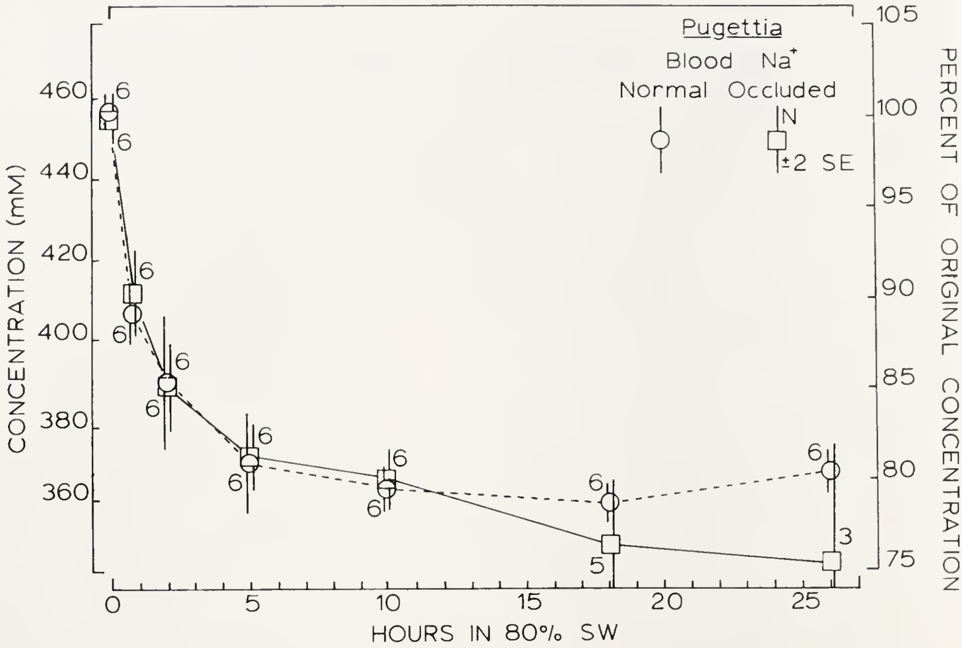

FIGURE 2. Changes in the blood concentration of sodium in normal and nephropore-occluded specimens of *Pugettia producta* transferred from 100 to 80% sea water. Normal (average weight, 104 g) and nephropore-occluded (89 g) crabs are indicated by open circles and squares, respectively. Note that during these experiments, some nephropore-occluded crabs died and that the concentration of the medium changed from 79.5 to 79.0% sea water.

$\log_e [((W_t/W_0) - 1)(1/t)]$ versus t, where t is time, and W_t and W_0 are the weights at times t and 0, respectively. In the case of normal crabs, W_t was sometimes less than W_0 and therefore the above logarithmic term was not defined. These data were coded by adding 0.01 to the term W_t/W_0 and regressed against the square root of time, in order to eliminate the significant deviations from linear regression which coding had introduced. In the case of nephropore-occluded crabs, it was unnecessary to code the data. Analysis of covariance indicates that the intercepts of the regressions for normal and nephropore-occluded specimens of *Pugettia* are significantly greater than those for normal ($P < 0.001$) and nephropore-occluded ($P < 0.05$) specimens of *Libinia*, respectively. No differences between the slopes in either pair of regressions were found. The antilog$_e$ of the intercept is equal to C in the above equation. At time 0 the first derivative is C (even after the data have been coded). Thus, the initial rate of weight gain is greater in specimens of *Pugettia*, and since this is an indication of the rate of water influx, it appears that *Pugettia* is more permeable to water than *Libinia* (see Cornell, 1979b).

In *Pugettia*, the urine production rate increased from about 6 to 30% bw/day during the first hour after transfer to 80% SW. After the first hour, urine production decreased rapidly to about 12% bw/day. The changes in urine production in *Libinia* are similar to those in *Pugettia*, although the data are based on only two animals. Urine production for these two crabs, expressed as the cumulative volume produced from the time of transfer from 100 to 80% SW, is shown in Figure 1.

Changes in hydrostatic pressure in Libinia

The urine production rate should be sensitive to changes in hydrostatic pressure. In 100% SW, the hydrostatic pressure in the ventral sinus was 7.5 ± 2.0 cm H_2O (mean ± SD) in nine specimens of *Libinia* (average weight, 135 g). Normal hydrostatic pressure is partly a function of muscle tone, and sudden increases of about 1.5 cm H_2O could be brought about by visual stimuli, such as sudden hand movements, or turning off the lights. Hydrostatic pressure measurements in three crabs exposed to 80% SW were made. In all crabs, maximum hydrostatic pressure occurred within the first hour of exposure to 80% SW. The average maximum and initial values were 14 and 7.5 cm H_2O, respectively. It took about 7 hr for the hydrostatic pressure to return to its original value.

Changes in the ionic composition of the blood of Pugettia

Sodium concentrations in normal and nephropore-occluded crabs transferred from 100 to 80% SW appear in Figure 2. The data for chloride is very similar to that for sodium. There is little difference between normal and nephropore-occluded crabs with respect to the changes in these two ions. In both groups of crabs the blood concentrations approached 80% of their initial values.

In normal crabs, the blood concentration of magnesium declined slowly (Fig. 3). After 26 hr it was less than 80% of its initial value. This was probably caused by the relative increase in the magnesium excretion rate, the rate being greater than 80% of the rate in 100% SW. In nephropore-occluded crabs, the decline in blood concentration was even slower. In this case it was expected that

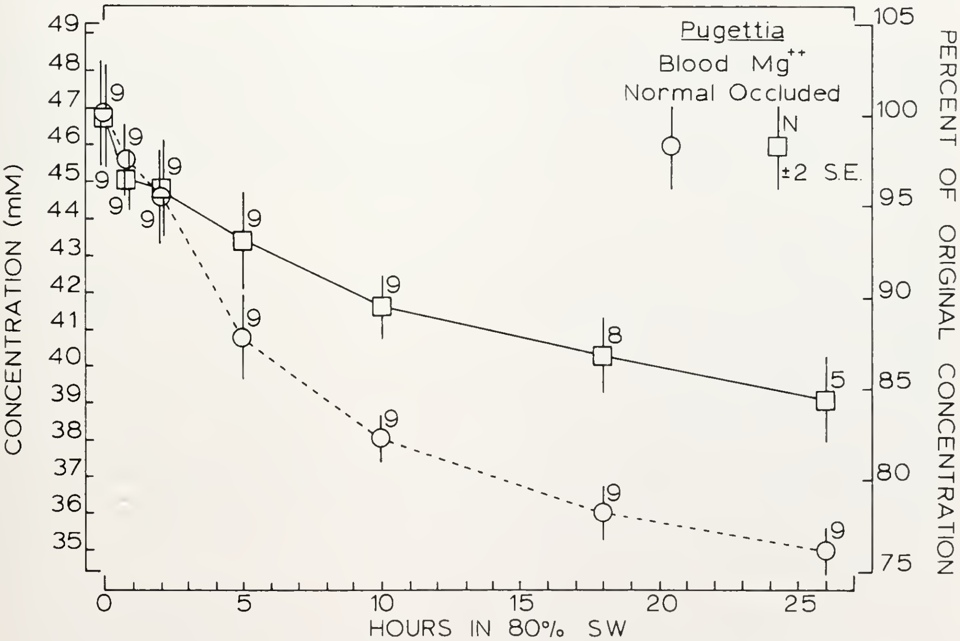

FIGURE 3. Changes in the blood concentration of magnesium in normal and nephropore-occluded specimens of *Pugettia producta* transferred from 100 to 80% sea water. Average weights are 97 and 92 g for normal and nephropore-occluded crabs, respectively. See Figure 2 for other details.

Table I

The rates of concentration change of ions in the blood of normal and nephropore-occluded specimens of Pugettia producta *transferred from 100 to 80% sea water. Rates were calculated from the equation* $f'(0) = -kB$ *which is the first derivative at time 0 of* $f(t) = A + B\exp(-kt)$, *where t is time, A is the concentration at time infinity, B is the change in concentration between time 0 and infinity, and k is a rate constant. A, B, and f(t) were estimated from the data, f(t) being estimated from the group of samples that was taken at the time when about half of the change in concentration had occurred, and k was calculated from these quantities. A relative rate was calculated by dividing the absolute initial rate by the initial concentration,* i.e., $A + B$. *An asterisk denotes that quantity A for calcium and potassium in nephropore-occluded crabs was estimated from that in normal crabs because of the secondary changes in these two ions (see text).*

Condition of nephropores	Ion	f(t) concn. at time t mM	t time hr	A final concn. mM	B concn. change mM	k rate constant 1/hr	$f'(0) = -kB$ initial rate of concn. change mM/hr	$\frac{kB}{A+B}$ relative initial rate of concn. change 1/hr
Normal	Cl^-	471	0.75	423	98	0.952	−93.3	0.179
	Na^+	406	0.75	363	93	1.03	−95.6	0.210
	Mg^{2+}	40.9	5.0	35.6	11.3	0.151	−1.71	0.0365
	Ca^{2+}	9.96	0.75	9.6	1.84	2.18	−4.00	0.350
	K^+	8.34	0.75	8.1	1.97	2.70	−5.32	0.529
Occluded	Cl^-	468	0.75	422	106	1.11	−118	0.224
	Na^+	414	0.75	358	97	0.732	−71.0	0.156
	Mg^{2+}	43.6	5.0	39.5	7.25	0.114	−0.826	0.0177
	Ca^{2+}	9.83	0.75	9.6*	1.96	2.86	−5.60	0.484
	K^+	8.38	0.75	8.1*	2.25	2.69	−6.04	0.585

the concentration would approach that of the medium, *i.e.,* 41.2 mM; but, at 26 hr it was significantly less than this, *i.e.*, 39.5 mM. This could indicate that magnesium is actively removed via extra-renal routes; however, such a conclusion does not seem warranted since by 26 hr a number of nephropore-occluded crabs had died and those remaining were physiologically distressed. It should be noted, however, that salts are concentrated in the gut of *Homarus americanus* when this animal is transferred to a dilute salinity (Dall, 1970).

In normal crabs, calcium declined to a concentration of about 83% of its initial value (Fig. 4). This is consistent with the fact that a considerable amount of calcium is bound to blood proteins (Robertson, 1953; Greenaway, 1976). In nephropore-occluded animals, there were secondary increases in calcium concentrations beginning at about 2 hr after transfer. It seems possible that calcium could be mobilized from the exoskeleton under these conditions. Potassium concentrations in normal animals changed much like those of sodium, except that the change took place at a greater rate. In nephropore-occluded animals there were also secondary increases in potassium, beginning at about 15 hr after transfer, which were correlated with the death of some crabs and which probably resulted from the breakdown of cells.

The means of the concentrations, at 0.75 hr after transfer to 80% SW, expressed as percentages of the initial concentrations, were compared in two single classification analyses of variance. *A priori* tests indicate that in normal crabs the relative concentration of magnesium is greater than ($P < 0.001$), and that for potassium is less than ($P < 0.001$), the relative concentrations of chloride, sodium and calcium. The results are similar in nephropore-occluded crabs, except that

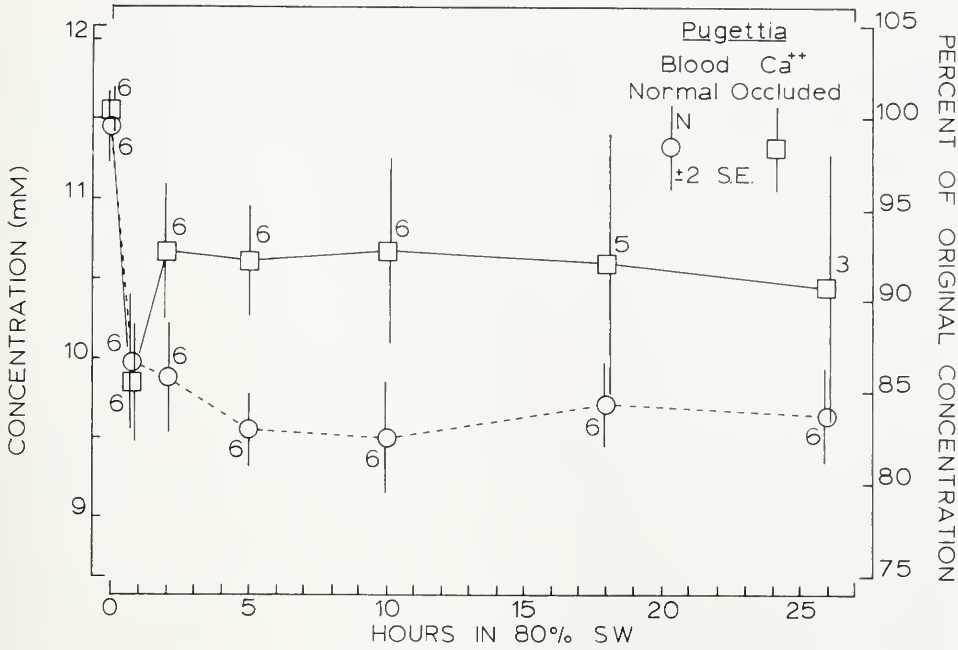

FIGURE 4. Changes in the blood concentration of calcium in normal and nephropore-occluded specimens of *Pugettia producta* transferred from 100 to 80% sea water. See Figure 2 for other details.

there is a difference among chloride, sodium and calcium ($P < 0.05$). Comparison of the relative initial rates of concentration change (Table I), for normal and nephropore-occluded crabs, suggests that the urine is the major route for magnesium loss, but that extra-renal routes are of major importance for the other ions. Some estimates of sodium and magnesium loss via the urine are shown in Table II.

DISCUSSION

Changes in volume and urine production, and diffusion of salts across the body wall are important factors in the short-term adaptation of *Libinia emarginata* and *Pugettia producta* to dilute salinity. Weight gain in these crabs is small when compared to that in some soft bodied animals, *e.g.*, *Phascolosoma* gains 22% bw when placed in 80% SW (Adolph, 1936); however, it serves temporarily to store a large portion of the influx of water (Fig. 1), thus lowering the demand on the antennal gland and reducing the potential hydrostatic pressure. At the time of transfer from 100 to 80% SW, if no weight gain occurred, urine would have to be produced at 25 and 15 times the normal rate in *Pugettia* and *Libinia*, respectively. Between the first and second hours after transfer, urine production accounts for most of the water influx, since urine production increases by a factor of about 5 and the concentration gradient is much reduced.

In both *Libinia* and *Pugettia* the sum of the cumulative volume of urine and the weight gain is less than the weight gain in nephropore-occluded animals (Fig. 1). This difference suggests that it is not possible to predict the change in urine production rate in this situation from the difference between the weight gain in normal and nephropore-occluded animals. This can probably be accounted for by the fact

TABLE II

Time-averaged rates of sodium and magnesium loss in the urine of specimens of Pugettia producta *(average weight, 118 g) transferred from 100 to 80% sea water. Standard deviations ($N = 5$) are included in parentheses below the means.*

Time period hr	Na^+ micromole/(g·hr)	Mg^{2+} micromole/(g·hr)	Urine flow rate μl/(g·hr)
0–2	3.97 (2.08)	0.539 (0.266)	8.97 (4.89)
2–5	2.19 (1.04)	0.289 (0.136)	5.30 (2.48)
5–10	2.03 (0.511)	0.281 (0.080)	5.28 (1.20)

that not only does the urine eliminate water, but since it is isosmotic to the blood, it also eliminates considerable quantities of salts. In the absence of urine production, these salts, particularly the less permeant ones, tend to remain in the blood where their osmotic activity causes a further increase in volume.

In *Libinia,* the increase in urine production can be correlated with an increase in hydrostatic pressure. However, urine production increased by a factor of about 5 while the hydrostatic pressure did not quite double. Unfortunately, it is not known how the hydrostatic pressure across the coelomosac is affected by the increase in hydrostatic pressure in the thoracic sinus. In *Carcinus,* the increase in hydrostatic pressure does not seem to totally account for the increase in urine production (Norfolk, 1978). Norfolk suggests that *Carcinus* might increase urine production by "anticipating" the change in internal conditions from the change in the external medium. It is not clear whether this is the case in *Libinia* and *Pugettia;* however, changes in hydrostatic pressure in *Libinia* do not seem to explain why urine production did not soon return to normal, since hydrostatic pressure did. There is evidence for the presence of diuretic hormones among the decapods (see Kamemoto and Tullis, 1972), and their presence in *Libinia* and *Pugettia* might explain why urine production did not immediately return to normal.

During the first two hours of exposure to 80% SW, direct estimates of the time-averaged rates of renal loss of sodium and magnesium are 4.0 and 0.54 micromole/(g·hr), respectively (Table II). These estimates are probably high because of the influence of the initial amounts of ions contained in the bladders. Independent estimates can be made by integrating the product of the blood concentration and the urine production rates represented by $f(t) = A + B\exp(-kt)$ and $g(t) = D + Ct\exp(-jt)$, respectively:

$$\int f(t)g(t)dt = ADt + \frac{BDe^{-kt}}{-k} - \frac{(jt+1)ACe^{-jt}}{j^2} - \frac{((j+k)t+1)BCE^{-(j+k)t}}{(j+k)^2}.$$

Definitions and values for A, B and k are found in Table I. D is the rate of urine production for crabs in 100% SW and the term $Ct\exp(-jt)$ was arbitrarily chosen to represent the increase in urine production of crabs exposed to 80% SW. When expressed in units of ml/(g·hr), values of D, C and j are 0.0025, 0.024 and 0.98, respectively, for the crabs in Table II.

Evaluating this integral for sodium and magnesium from 0 to 2 hr and dividing the results by 2 hr, so that they are expressed in a form comparable to that in Table II, indicates that sodium and magnesium were filtered from the blood at 3.9

and 0.44 micromole/(g·hr), respectively. It is also necessary to account for the rate at which magnesium is actively concentrated in the urine. In 100% SW, magnesium is 24 mM greater in the urine than in the blood, while the urine production rate is 0.0025 ml/(g·hr) (Cornell, 1979a). Thus, magnesium is concentrated in the urine at 0.06 micromole/(g·hr) and it leaves via the urine, during the first 2 hr, at $0.44 + 0.06 = 0.50$ micromole/(g·hr). These estimates are not much lower than those obtained by direct measurement which suggests that the bladder volumes were not particularly large in these animals.

Total rates of ion loss were not measured, but some estimates can be made. It will be assumed that the ion space is constant, since the maximum volume change in normal specimens of *Pugettia* is small (about 5% of the ion space). Twenty-five percent bw can be assumed to be a minimum estimate for the ion space, since this is the inulin space in intermolt specimens of *Pugettia* (Born, 1970). Thirty-five percent bw can be considered a reasonable upper limit. A few examples from studies on *Carcinus maenas*, which is approximately isotonic to the medium when in 100% SW, will suffice: when the total body chloride was assumed to be in the blood, its volume was calculated as 36% bw (Webb, 1940); the calcium space is 25% bw (Greenaway, 1976); the inulin space is 19% bw (Binns, 1969). No doubt some inorganic ions of intracellular origin enter the blood during exposure to 80% SW, but the amounts are small. Changes in free amino acids account for the major change in intracellular osmotic concentration when *Libinia* is transferred to 50% SW (Gilles, 1970). Freel (1978) has shown that the intracellular concentrations of potassium, and calcium plus magnesium, in the muscles of a number of decapods, are independent of extracellular concentration. However, intracellular sodium and chloride concentrations were found to be a function of the extracellular concentration and thus some sodium of intracellular origin probably entered the blood. But, since the intracellular sodium concentration is low (about 20 mM), the following results can be affected by no more than 5%.

The time-averaged rate of ion loss can be estimated as $(1/t)VB(1 - \exp(kt))$; where t is time, V is the weight-specific ion space (*e.g.*, 0.35 ml/g), B is the change in ion space concentration between times 0 and infinity, and k is a rate constant. Values for B and k are found in Table I. For normal animals, assuming an ion space of 35% bw, the rates of sodium and magnesium loss are 14 and 0.51 micromole/(g·hr), respectively. If 25% bw is assumed, these rates are 10 and 0.36 micromole/g·hr), respectively. Thus, the renal rate of sodium loss is less than 40% of the total, while the renal rate of magnesium loss must nearly equal the total rate of loss. Most studies of osmoregulating decapods have shown that renal salt loss, that is of sodium or chloride, is less than 25% of the total (*Eriocheir*, Krogh, 1938; *Carcinus,* Shaw, 1961; *Astacus,* Bryan, 1960; *Rithropanopeus,* Smith, 1967; *Potamon*, Harris, 1975; *Paranephrops*, Wong and Freeman, 1976; *Uca,* Baldwin and Kirschner, 1976a, b; *Callinectes*, Cameron 1978).

One question of interest is what happens when animals are transferred to a more concentrated medium. In this case, urine production would be of little importance unless produced at negative rates. When *Pugettia* was transferred to 100% SW, after acclimation to 80% SW for five days, there were no differences between the weight changes in normal and nephropore-occluded crabs. However, normal specimens of *Pugettia* and *Libinia* show larger absolute weight changes when transferred back to 100% SW (1.8 and 0.75% bw, respectively) than when transferred to 80% SW (1.4 and 0.56% bw, respectively). These data suggest that extra-renal routes are of primary importance and reflect the asymmetry of the transfers caused by the presence or absence of the renal route of ion movement. Thus in *Libinia* and *Pugettia,* volume change and diffusion of salts across the body wall are factors of general importance whenever these animals are exposed to a change in salinity. Urine production is an important factor when animals are exposed to a dilute salinity, but seems to be of no importance during an exposure to a concentrated salinity.

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SUMMARY

1. *Libinia emarginata* and *Pugettia producta* respond to 80% sea water (SW) in similar ways. In both species, during the first hour of exposure to 80% SW an increase in volume, 0.56 and 1.4% body weight (bw) for *Libinia* and *Pugettia* respectively, accommodates the major portion of the influx of water.

2. Urine production increases from about 6 to 30% bw/day in both species during the first hour of exposure to 80% SW. Elimination of salts and water via the isosmotic urine helps to decrease the potential for an increase in hydrostatic pressure. The hydrostatic pressure in *Libinia* changes from 7.5 to 14 cm H_2O during the first hour of exposure to 80% SW.

3. In *Pugettia*, most of the ions in the blood decrease at a rate proportional to the concentration difference between the blood and the medium. The ordered rates of concentration changes are as follows: potassium > calcium > sodium $\simeq$ chloride >> magnesium. Blocking the nephropores has a greater effect on the rate of magnesium concentration change than on the other ions. With the exception of magnesium, all other ions which were measured are lost from the blood mainly via extra-renal routes.

4. During the second hour of exposure to 80% SW, the volume of both species declines, probably as a result of the increase in urine production and the reduction in the rate of water influx caused by the reduction in the concentration gradient. By contrast, the volume of nephropore-occluded crabs of both species continues to increase, reaching at least 5% bw after 4 hr of exposure to 80% SW.

5. When transferred from 80 to 100% SW, specimens of *Libinia* and *Pugettia* lose 0.75 and 1.8% bw, respectively. Blocking the nephropores has no effect in this case and the gain in salts must take place entirely across the body surface, since urine flow is unidirectional.

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INDUCED HOST ODOR ATTRACTION IN THE PEA CRAB *PINNOTHERES MACULATUS*

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Chemoreception often plays an important part in the initiation and maintenance of marine symbiotic relationships (Davenport, 1955, 1966; Dales, 1966; Castro, 1978), including pinnotherid decapod crustaceans (*Dissodactylus mellitae*: Johnson, 1952; Gray, McCloskey, and Weihe, 1968; *Pinnixa chaetopterana*: Davenport, Camougis, and Hickok, 1960; *Pinnotheres maculatus*: Sastry and Menzel, 1962; *Opisthopus transversus*: Webster, 1968). Sastry and Menzel (1962) found that adult specimens of *P. maculatus* removed from the bay scallop *Argopecten irradians concentricus* are attracted to the odor of live specimens of both *Argopecten* and *Atrina rigida* (pen shell), but not to odor from their shells alone. This implies that the attractant is a metabolic product of the living host. Other sensory factors may be important in the associations of pinnotherids with their hosts. Phototactic responses may direct larvae of *P. maculatus* (Welsh, 1932) and other pinnotherids (Miyake, 1935) toward their hosts at the end of the planktonic stage. Rheotaxis and thigmotaxis can be used by adult specimens of *P. maculatus* in host location (Eidemiller, 1969).

Host location is important at several stages in the life cycle of *P. maculatus*. The planktonic zoeal and megalopal stages are followed by a first crab stage ("invasive stage"), at which time crabs leave the plankton and invade hosts. Following several molts within the host, the "hard stage" develops. Males and females of this stage leave their hosts, engage in an open water copulatory swarming, resettle into hosts, and pass through four posthard stages before reaching the adult instar (Pearce, 1964, 1966). Host location would therefore be necessary at the invasive and hard stages, as well as at any stage when the host might die. There are fourteen reported host species for *P. maculatus*; of these, ten are bivalve species, including the blue mussel *Mytilus edulis*, the bay scallop *Argopecten irradians concentricus,* the ocean scallop *Placopecten magellanicus,* the horse mussel *Modiolus modiolus,* and *Modiolus americanus* (Williams, 1965).

Search images are said to be involved in selective predation (Tinbergen, 1960). Search images are formed by a predator following recent encounters with a prey species, resulting in an improved ability of the predator to detect that prey species (Krebs, 1973). Search images may involve selective attention for certain stimulus objects (Curio, 1976). Although work has concentrated on visual predators, there is evidence that chemical search images may be developed by predators that use their chemical senses to a greater degree (Atema, 1977, 1980). Search images may also be used in symbiotic associations by a symbiont that must actively search for its host.

The present investigation examines chemically mediated host location in *Pinnotheres maculatus*. The analyses include: a comparison of the effects of odors from four bivalve species on searching behavior of the crabs; a determination of the location of the principal chemoreceptors used in host location; and an analysis of the importance of host induction ("olfactory conditioning": Thorpe and Jones, 1937; "ingestive conditioning": Wood, 1968; "host induction": Jermy, Hanson, and Dethier, 1968) in altering host preferences. This is discussed in light of the concept of chemical search images (Atema, 1977, 1980; Atema, Holland, and Ikehara, 1980).

MATERIALS AND METHODS

Subadult posthard stages (Pearce, 1964) and adult male and female specimens of *Pinnotheres maculatus* were collected from specimens of *Mytilus edulis* in the Woods Hole, Massachusetts, region. Their responses to odors from *Mytilus edulis, Argopecten irradians concentricus, Placopecten magellanicus*, and the ribbed mussel *Modiolus demissus* were tested. Of these four species, only *M. demissus* has never been reported as a host for *P. maculatus* (Williams, 1965).

The experimental apparatus was a plastic circular choice maze, modified from the design of Bartel and Davenport (1956) (Fig. 1). Odors were prepared for each species in 4-liter beakers by adding the number of live intact bivalves necessary to constitute 50 g of soft tissue wet weight per one liter of sea water. Odors and/or sea water dripped into the six radial chambers. This presented the crabs with six simultaneous stimuli from which they could make a choice. Each trial consisted of placing between six and thirty crabs in the central chamber. They were given 10 hr to enter the radial chambers, after which the trial was stopped and the crabs' distribution recorded.

Three types of tests were performed: control tests, where all six radial chambers received sea water; single-stimulus experimental tests, where odor dripped into only one of the radial chambers, and sea water into the other five chambers; and two-stimuli choice experimental tests, with four sea water radial chambers, and two chambers receiving odors, each odor from a different bivalve species. In choice experiments, the two chambers containing odors were always diametrically opposite each other. The apparatus was rotated 60° after each trial.

To determine the sites of the chemoreceptors used in host location, either antennules (A1) or antennae (A2) were ablated at least two days before the crabs were tested.

Host induction experiments were performed to analyze the degree of plasticity of the chemoresponses of these crabs. In these experiments, only adult crabs removed from *Mytilus* hosts were used. They were divided into two groups: induced crabs, which were placed in one-liter bowls with three individuals of *Argopecten*; and non-induced crabs, which were placed in identical bowls with sea water only. During the induction process, the water and scallops were changed two to three times per week, and crabs were free to crawl into or onto the scallops. The responses of these crabs in two-stimuli choice experiments (using *Mytilus* and *Argopecten* odors) were tested before induction and at weekly intervals for 5 weeks.

Two behavioral responses were analyzed for each trial run: chemically stimulated movement, and direct location of the odor source. Chemically stimulated movement (excitation) is defined as any upstream movement in response to the test solution and is measured as the percentage of crabs that entered any of the six radial chambers ("per cent excited"). It was not determined whether this movement was a rheotaxis, an increase in random movement, or both. Location of odor (attraction) is defined as the ability of crabs to find the source of the test solution and is measured as the percentage of all excited crabs that entered the chamber containing the odor ("per cent attracted").

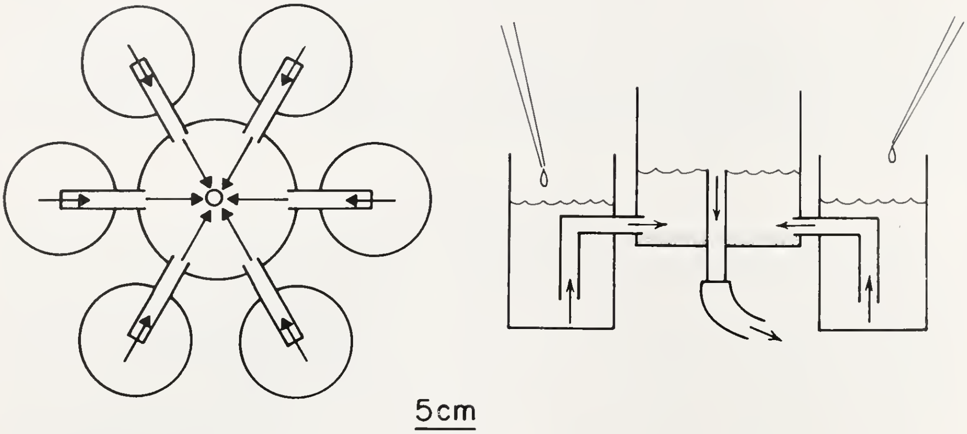

FIGURE 1. Circular choice maze. The maze consists of six radial chambers connected via L-tubes to a central chamber. The system was run under constant illumination, salinity (33%), and temperature (14–18° C), with sterilized sand (3 hr at 290° C) covering the bottom of the central chamber. Water dripped into each of the radial chambers at a rate of 2 to 3 drops per sec. Water flowed from each of the radial chambers into a central chamber, which drained by a central standpipe. Arrows indicate the direction of water flow.

Statistical analyses are based on the assumption that the distribution of crabs in the radial chambers is related to their ability to sense a change in the chemical environment. Chi-square values were calculated for each trial to test if there was a preference for the chamber with the body odor. Heterogeneity Chi-square analyses were performed to check the validity of combining all the replicate trials within each treatment group so that an overall Chi-square test could be performed. All tests met this condition, except for the response to *Modiolus* odor, in which female crabs showed an attraction, but males and subadults did not. Chi-square tests were then performed for the compiled data using the 0.05 level of significance.

RESULTS

The control tests demonstrate that the crabs had no preference for any single radial chamber ($N = 140$, $\chi^2 = 8.96$, $P > 0.10$). Single-stimulus experimental tests show that intact crabs removed from specimens of *Mytilus* were highly attracted to *Mytilus* host odor and did not respond significantly to odor from *Argo-*

TABLE 1

Effects of bivalve odors on P. maculatus *searching behavior.*

Stimulus	% Excited	% Attracted	N	χ^{2}*	P
Mytilus odor	90.7	55.6	129	127.40	<0.001
Placopecten odor	85.3	19.7	95	0.56	>0.25
Argopecten odor	87.6	15.3	97	0.12	>0.50
Modiolus odor					
Female crabs	84.4	42.1	45	17.73	<0.001
Male and S crabs	70.2	17.0	67	0.01	>0.90

* Chi-square values determined by comparing number of crabs attracted to the radial chamber with bivalve odor and number of crabs attracted to the other five sea water radial chambers against the distribution expected from random movement. S = subadult posthard stage crabs.

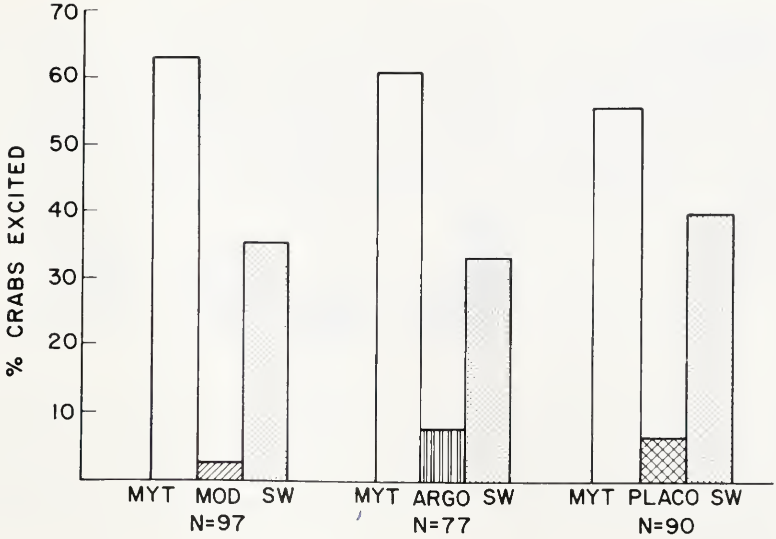

FIGURE 2. Preference of *P. maculatus* for *Mytilus* host odor in two-stimuli choice experiments. Chi-square tests show significant differences ($P < 0.001$) for all three tests in which the number of crabs attracted to *Mytilus* host odor and number of crabs attracted to odor from the other bivalve species are compared to the distribution expected from random movement. MYT = crabs attracted to *Mytilus* chamber; ARGO = crabs attracted to *Argopecten* chamber; PLACO = crabs attracted to *Placopecten* chamber; MOD = crabs attracted to *Modiolus* chamber; SW = crabs attracted to the four sea water chambers.

pecten or *Placopecten*; only the female crabs responded to *Modiolus* odor (Table I). In the two-stimuli choice experiments, crabs from *Mytilus* hosts strongly preferred *Mytilus* odor over odor from any other bivalve species tested ($P < 0.001$) (Fig. 2).

Ablation studies were performed to determine the sites of chemoreceptors used in host location. Trauma caused by appendage ablation is negligible, since intact crabs and crabs without antennules responded similarly in control tests: both groups show an equal degree of random movement (77.9 and 75.0% excitation, respectively), and both groups show random distribution in the six radial chambers ($P > 0.10$ and $P > 0.50$, respectively). Intact crabs and crabs without an-

TABLE II

Effects of ablations on chemically stimulated searching behavior.

Appendages removed	% Excited†	% Attracted	N	χ^2*	P
None	90.7	55.6	129	127.40	<0.001
Antennae (A2)	96.6	40.4	59	23.02	<0.001
Antennules (Al)	72.8	22.0	81	1.23	>0.25

Single-stimulus experiments, using *Mytilus* host odor as the stimulus.

* Chi-square values determined by comparing number of crabs attracted to *Mytilus* chamber and number of crabs attracted to the other five sea water chambers against the distribution expected from random movement.

† A one-way ANOVA with Scheffé's Multiple Comparison Procedure (using % excited values normalized by arcsine transformation) demonstrates that host odor caused increased movement (excitation) in intact and A2-less crabs, but not A1-less crabs ($P < 0.05$).

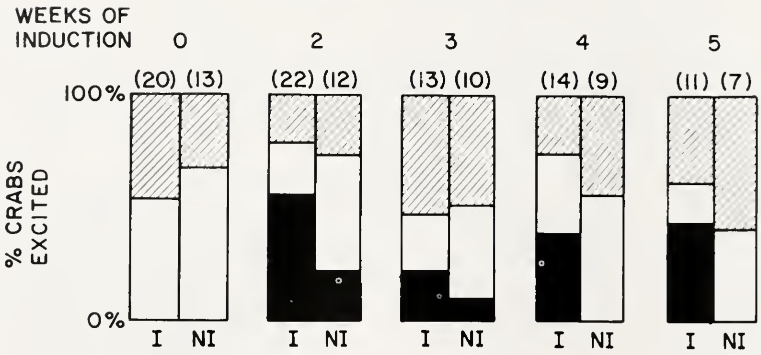

FIGURE 3. Induction of crabs from *Mytilus* to *Argopecten*. At weeks four and five, analysis by 2×2 contingency tables demonstrates a significant difference ($P < 0.05$) between number of induced crabs and number of non-induced crabs choosing the *Argopecten* chamber. Number in parentheses is the total number of crabs excited. I = Induced crabs; NI = Non-Induced crabs. Shaded areas represent crabs attracted to *Mytilus* chamber; open areas represent crabs attracted to the four sea water chambers; solid areas represent crabs attracted to *Argopecten* chamber.

tennae were significantly attracted to host odor, whereas the crabs without antennules were not (Table II). In addition, a one-way analysis of variance (ANOVA) demonstrates that host odor also caused an increased excitation in intact crabs and in crabs without antennae, but not in the crabs without antennules (Table II). Thus, antennules are the primary sites of chemoreceptors that detect *Mytilus* host odor, resulting in both increased general movement and source location.

Induction experiments were performed to explain why crabs found in *Mytilus* hosts do not respond to odors from other bivalves in which pea crabs are known to inhabit. These experiments (Fig. 3) reveal that experience with a new host significantly altered the olfactory response of adult crabs to the host odor. Non-induced crabs maintained a high preference for *Mytilus* odor, with little response to *Argopecten* odor. Induced crabs, on the other hand, showed an increased responsiveness to *Argopecten* odor after two weeks of induction. By the fourth and fifth weeks of induction, the difference in the responses of induced and non-induced crabs to *Argopecten* odor became statistically significant ($P < 0.05$). However, even with this increased response to *Argopecten* odor, a large proportion of the crabs still responded to the *Mytilus* odor.

DISCUSSION

This investigation demonstrates that there are chemoreceptors on the antennules of *P. maculatus* that elicit searching behavior in the presence of odor from the host, *Mytilus edulis*. These crabs, however, failed to respond to odor from two other bivalve species, *Argopecten irradians concentricus* and *Placopecten magellanicus*, and only the female crabs seemed to be attracted to *Modiolus demissus* odor (Table I). The latter observation cannot, at present, be explained. Also, in choice experiments, the crabs showed a strong preference for *Mytilus* odor over the odor from other potential hosts (Fig. 2). These results are different from those of Sastry and Menzel (1962), who found that *P. maculatus*, removed from *Argopecten*, is attracted to odor from both *Argopecten* and *Atrina*, and when given a choice between the two bivalves, is equally attracted to both. This discrepancy could be explained by several factors. Different populations of pea crabs could be responding differentially to the same stimulus due to slight differences in olfactory receptors, as has been shown in the oriental fruit fly (Metcalf, Metcalf, Mitchell and Lee, 1979). Alternatively, odors of different populations of *Argopecten* may be qualitatively and/or quantitatively different (Bayne and Scullard, 1977), affecting the responses of the crabs. In addition, the odors of *Argopecten* and *Atrina* may be more similar to each other than are the odors of *Mytilus* and the other bivalves studied here.

Antennular chemoreceptors accounted for most of the response of the pea crabs to *Mytilus* host odor (Table II). Antennules are known by behavioral and neurophysiological studies to be important in host location for the commensal shrimp *Betaeus harfordi* and *B. macginitieae* (Ache and Case, 1969; Ache and Davenport, 1972; Ache, 1975). For pea crabs, hosts provide not only shelter but a constant food source; therefore, host odor might also be considered a feeding stimulus.

A plausible explanation for the observation that adult crabs show little attraction to odors from hosts which conspecifics are known to invade is that once a crab inhabits a host, it develops a stronger chemical attraction to the odor of that host. The induction experiments, designed to test this hypothesis, demonstrate that olfactory preferences of adults can be altered. After two weeks of induction to *Argopecten*, there was an increased responsiveness to *Argopecten* odor; this increase became statistically significant after four weeks (Fig. 3). Gray *et al.* (1968) reported changes in olfactory responses of the pinnotherid crab *Dissodactylus mellitae* to host odors after only one or two days of "acclimation" to a new host species. Modification of olfactory responses due to experience with a host or host odor has been reported for a variety of other symbiotic invertebrates (parasitic wasp *Nemeritis canescens*: Thorpe and Jones, 1937; tobacco hornworm *Manduca sexta*: Jermy *et al.*, 1968; polynoid polychaete *Arctonoe pulchra*: Dimock and Davenport, 1971).

The mechanism for such olfactory plasticity used in searching behavior could be described as chemical search image formation. The concept of search images was first discussed by von Uexküll (Uexküll and Kriszat, 1934), and later developed by Tinbergen (1960) to explain prey selection by the titmouse *Parus major*. Tinbergen described search image formation as a central perceptual change, involving improvement of the ability of the predator to detect the prey. As this bird is a visual predator, the term "visual search image" was adopted. Chemical search images are functionally similar, but are more important for animals relying on input from chemical senses, such as pea crabs. When the chemical search image of the animal is matched by the "chemical picture" of the environment, searching behavior may be initiated (Atema, 1977, 1980). It was recently shown that tuna change their olfactory excitability to specific prey odors depending on recent feeding experience. Modifiable chemical search images could describe the observed effects and appear useful in the tuna's behavioral ecology (Atema *et al.*, 1980). Home stream recognition by salmon (Scholz, Horrall, Cooper, and Hasler, 1976) could be considered a classical case of the use of a chemical search image.

This chemosensory plasticity would be adaptive considering that *P. maculatus* is a host-generalist, with fourteen host species reported (Williams, 1965). Although the chemosensory behavior of larval pea crabs is presently unknown, it might be expected that the "invasive stage" (Pearce, 1964, 1966) of this host-generalist symbiont can respond to a variety of chemical signals found in many potential hosts. Similar cases are known for other crustaceans: the settling stage of the symbiotic brachyuran *Echinoecus pentagonus* responds to chemical cues from its host urchin *Echinothrix calamaris* as well as to odors from the closely related *E. diadema*; larval barnacles (*Balanus balanoides* and *Elminius modestus*) settle in response to proteins in the epicuticle of many barnacle and other arthropod species (Crisp and Meadows, 1962). After finding a host, *P. maculatus* can become conditioned to this host species, such that the response to the odor of this species increases. Such a change in olfactory response has been demonstrated here for adult specimens of *P. maculatus*. This phenomenon would be important in host reinvasion which can occur in various life stages. For example, there would be an enhanced likelihood of reinvasion by the hard stage following copulatory swarming, and by any stage following damage or death to the host.

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SUMMARY

1. The pea crab *Pinnotheres maculatus* is a host-generalist symbiotic brachyuran. When subadult posthard and adult crabs are removed from the bivalve *Mytilus edulis, Mytilus* host odor increases their nonspecific movement as well as their location of the odor source. Odors from other previously recorded host species, however, generally do not affect their searching behavior.

2. Such response specificity may be due to olfactory induction to their host. Adult crabs removed from the blue mussel *Mytilus edulis* can be induced to respond to odor from the bay scallop *Argopecten irradians concentricus*. Such induction supports the plastic "chemical search image" concept.

3. Antennules are the principal sites of chemoreceptors used in host location.

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CHEMICAL CAMOUFLAGING AND BEHAVIORAL DEFENSES AGAINST A PREDATORY SEASTAR BY THREE SPECIES OF GASTROPODS FROM THE SURFGRASS *PHYLLOSPADIX* COMMUNITY

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The marine angiosperm *Phyllospadix* (surfgrass) commonly forms luxuriant beds in the lower rocky intertidal zone of the Pacific coast of North America. These beds provide a spatially heterogeneous substratum that supports a variety of inhabitants. Three conspicuous inhabitants of *Phyllospadix* beds along the coast of central California are the gastropods *Lacuna marmorata* Dall (a mesogastropod), *Alia carinata* (Hinds) (= *Mitrella carinata*; a neogastropod), and *Notoacmea paleacea* (Gould) (an archeogastropod). *Lacuna*, a herbivore, and *Alia*, an active, microcarnivore or scavenger, both occur widely on low intertidal rocks and algae, as well as on *Phyllospadix*. In contrast, the limpet *Notoacmea paleacea* occurs solely on *Phyllospadix* spp. This stenotopic limpet, which feeds directly on the epithelial layers of the surfgrass blade, possesses a striking series of morphological and behavioral adaptations for living on the narrow blades of surfgrass (Yonge, 1962; Fishlyn, 1976, and in preparation). Of particular interest here is the precise fit of the limpet's parallel-sided shell to the surfgrass blade, which enables the limpet to clamp down tightly on the blade surface if disturbed. All three gastropods are small: average size of *Lacuna* is about 4 mm, of *Alia*, about 8 mm, and of *Notoacmea*, about 6 mm.

The *Phyllospadix* beds provide a partial refuge against predation for these three small gastropods, and for other inhabitants, because the thin, floating blades are inaccessible to large, heavy, benthic predators such as the seastar *Pisaster ochraceus* (Brandt). However, the small (average diameter: 3 cm), agile seastar *Leptasterias hexactis* (Stimpson) is well suited for life on and among the blades of *Phyllospadix*, and it is a common resident of the surfgrass beds. In contrast to *Pisaster*, which possesses short, muscular tube feet, capable in concert of pulling apart its prey, *Leptasterias* possesses long, highly maneuverable (but relatively weak) tube feet. In the *Phyllospadix* beds, *Leptasterias* uses these tube feet to capture and ensnare small prey, which are then passed to the mouth. As the seastar moves along the surfgrass blade, the long tube feet are waved back and forth thus further extending the effective range of prey capture. Also occurring in surfgrass beds with *Leptasterias*, but far less frequently, is the larger seastar *Patiria miniata* (Brandt). *Patiria* is a particulate feeder and general omnivore (MacGinitie and MacGinitie, 1968; Sutton, 1975); it does not feed on live gastropods in the surfgrass beds. In contrast, *Leptasterias* is known to be an important predator of small gastropods when it occurs on rocky substrata (Menge, 1972).

Molluscan defensive adaptations against predators are manifested in many forms. Ansell (1969) has reviewed many of these adaptations and has classified them broadly as passive or active. Passive adaptations include morphological, physiological, and behavioral characteristics, such as the possession of a protective shell, camouflage, or nocturnal foraging pattern. These may reduce predation but are not released by the immediate presence of the predator. Active adaptations, which are primarily behavioral, are released specifically by the presence of the predator. In the marine environment, active defensive adaptations often are triggered by chemicals (Feder and Christensen, 1966; Feder, 1972; Mackie and Grant, 1974; Phillips, 1978). Chemically-mediated defensive behaviors may be triggered by waterborne chemicals, emanating from distant predators, or they may be triggered by actual contact with the predator. Some gastropods give qualitatively different responses to distant predators (or to water "scented" by the predator) than they do to contact with the same predator (Szal, 1970; Phillips, 1975, 1977).

This paper examines defensive adaptations of *Lacuna*, *Alia*, and *Notoacmea*. Responses to waterborne chemicals emanating from the predatory seastar *Leptasterias* are described in the laboratory and in the field, as are responses to contact with this predator. A possible case of chemical camouflage is reported.

Materials and Methods

All animals used in this study, except the limpet *Notoacmea insessa* (Hinds) were collected at low tide from *Phyllospadix scouleri* (Hooker) in Horseshoe Cove, adjacent to the Bodega Marine Laboratory, Bodega Bay, California. Specimens of *N. insessa* were collected nearby from stipes of the boa kelp *Egregia menziesii* (Turner) Areschoug. Animals were held in running sea water until use, which was within one week of collection. Preliminary behavioral observations were made during fall, 1976. Final behavioral experiments were conducted at the Bodega Marine Laboratory from October to December, 1978.

Behavioral observations

Responses to waterborne chemicals emanating from living seastars were examined initially in the laboratory. For these experiments, three kinds of test water were prepared, starting with 800 ml of sea water. Nothing was added to the first beaker, which served as a seawater control; one specimen of *Patiria miniata* (48.4 g wet weight) was added to the second; and 10 to 12 specimens of *Leptasterias* (11.0 g wet weight total) were added to the third. The three beakers were placed in a seatable and kept cold with running sea water (12° C) for approximately 2 hr before use. This procedure produced water "scented" by *Patiria* and *Leptasterias* (*i.e.*, "scented water" or SCW). For experiments, gastropods were attached, or allowed to attach, to blades of *Phyllospadix scouleri*. Initially, attached snails were oriented in all directions (up, down, and sideways). Adjustments were made to position the snails in mid-blade areas, so they had equal opportunity to move up or down. Water to be tested was poured into a 600-ml beaker and let stand for 1 min. Then, the blades with attached animals were lowered vertically into the test water. Typically, 5 to 10 snails of one species were tested at one time using 5 blades of surfgrass mounted in a plastic holder that rested on the top of the beaker. A wide range of sizes of snails, from smallest to largest available, was used. Observations were made continuously for the first minute, and then at 1-min intervals for an additional 4 min. Data presented here are at the 3-min interval, because a substantial percentage of snails (*Lacuna* and *Alia*) began to move out of the beaker after 3 min.

Responses to contact with seastars also were examined in the laboratory. Snails were placed on a mat of *Phyllospadix scouleri* on the bottom of a shallow glass bowl (20-cm diameter); the snails were covered with water, which was changed frequently. The *Phyllospadix* mat also was changed between testings of each species. Contact was made either with a blunt probe, tube feet of *Patiria miniata*, or tube feet of *Leptasterias hexactis*. Whole seastars were used, and two or more tube feet usually touched the gastropod. Responses were observed with a dissecting microscope mounted on a swing arm.

Field observations were made in Horseshoe Cove during lower low water in a large, surfgrass-filled, tide pool that received some flushing by waves even during minus tides. Individual *Leptasterias hexactis* were located *in situ*, and notice was taken of the behavior of snails on nearby surfgrass. Distances were measured between the seastar and the surrounding snails. Moving specimens of *Leptasterias* were followed, and the behavioral and predatory outcomes of contacts with snails were observed. After observations of an individual seastar in the tidepool, the seastar was turned over and examined to determine what it had been eating. Since *Patiria miniata* was not as common in the study area, encounters between snails and this seastar were staged by placing a seastar near cluster of snails.

Biochemical analyses

Fresh samples of *Phyllospadix scouleri* (11 g), shells of *Notoacmea paleacea* (0.48 g), soft parts of *N. paleacea* (0.23 g), shells of *Notoacmea insessa* (0.50 g), and soft parts of *N. insessa* (0.46 g) were processed by standard techniques (Mabry, Markham, and Thomas, 1970; Harborne, 1973; Harborne, Mabry, and Mabry, 1975; Swain, 1976).

Specimens of *Notoacmea paleacea* were starved for 48 hr before use in order to clean the gut of residual plant material, and shells clean of epizoics were used. All samples (*Phyllospadix* and limpets) were macerated with mortar and pestle, initially at room temperature; then boiling 95% ethanol was added, and maceration continued. The tissue was extracted in ethanol overnight and centrifuged at 18,000 rpm for 30 min to remove precipitated proteins. The supernate was extracted with hexane to remove chlorophyll, carotenoids, and lipids. Samples of the alcoholic polar fraction were withdrawn for ultraviolet spectrophotometry and paper chromatography. Spectral data were obtained with a Perkin-Elmer 550 spectrophotometer operating over the range from 600 to 190 nm.

Two-dimensional, ascending paper chromatography was carried out using Whatman No. 1 paper. The first solvent was the upper phase of BAW (n-butanol: acetic acid: water, in a 4 : 1 : 5 ratio), and the second solvent was 15% acetic acid. Spots were visualized in daylight and under ultraviolet light (254 nm) before and after fuming with ammonia vapor. Selected spots were eluted in a small volume of 50% ethanol for 24 hr, and the UV spectrum of each eluate was determined.

RESULTS

Defensive responses of Lacuna marmorata

When specimens of *Lacuna marmorata* were placed in water from a beaker that previously had contained *Leptasterias hexactis* (*i.e., Leptasterias*-SCW), the snails' behavior changed dramatically from the resting state. *Lacuna* often reacted within 5 seconds of being placed in the stimulatory water, and within 30 seconds, all snails were reacting. Most commonly, the first response was to elevate the shell over the foot, a behavior aptly described as "mushrooming" by Bullock (1953) in reference to the defensive responses of another gastropod, the limpet *Collisella*

TABLE I

Responses of Lacuna marmorata *to waterborne chemicals emanating from distant specimens of* Patiria *and* Leptasterias *in the laboratory. Snails were considered reacting if they rotated the shell and waved the cephalic tentacles within 3 min. Simultaneously, snails were scored for upward or downward movement without regard to whether or not they were showing shell-rotation behavior.*

Stimulus	Number of snails		Number of snails		
	Reacting	Not reacting	Moving up	Moving down	Stationary
Control (N = 60)	0	60	1	0	59
Patiria (N = 60)	2	58	12	0	48
Leptasterias (N = 60)	60	0	19	3	38
Leptasterias (field) (N = 43)	41	2	—	—	—

limatula. Next, the shell and visceral mass began to swing violently back and forth through an arc of 360° while the two cephalic tentacles moved up and down rapidly and in unison. More than half of the reacting snails remained in place on the blade of surfgrass as they rotated the shell and waved their tentacles. The others ran, still twisting, and some of these climbed out of the water. This series of responses was also seen in the field when *Leptasterias* was nearby.

Two independent aspects of *Lacuna* behavior in control sea water, *Patiria*-SCW, and *Leptasterias*-SCW were examined quantitatively and are presented in Table I. These aspects are first, the number of snails that responded with shell rotation and tentacle waving, and second the number of snails that moved up, down, or remained stationary. In control sea water, none of the 60 snails tested responded with shell rotation or synchronous tentacle waving during the 3-min test period. In *Patiria*-SCW, 2 of 60 snails responded, and in *Leptasterias*-SCW, all 60 responded. A series of field observations of *in situ Leptasterias* confirmed the responses of *Lacuna* to *Leptasterias*: 41 of 43 specimens of *Lacuna* within 9 cm of seastars were responding with shell rotation and tentacle waving.

Directional movement along the blade of surfgrass was more frequent in seastar-scented water (Table I). Only 1 of 60 snails moved in control water, whereas 12 snails moved in *Patiria*-SCW ($\chi^2 = 8.63$; $P < 0.005$) and 22 moved in *Leptasterias*-SCW ($\chi^2 = 21.52$; $P < 0.001$). A greater number of snails moved in the presence of *Leptasterias* than in the presence of *Patiria* scent, but this difference, while probably real in light of the data on the shell-rotation response, was not statistically significant ($\chi^2 = 3.32$; $0.1 > P > 0.05$).

The combined response of *Lacuna* elicited by *Leptasterias* (*i.e.*, shell-rotation, tentacle waving, and movement) differed markedly from that elicited by *Patiria*. Within the first 30 seconds of being placed in *Leptasterias*-SCW, all 60 specimens of *Lacuna,* although not yet moving, were vigorously twisting their shells and waving their cephalic tentacles; and within 1 min, 14 of the 60 specimens of *Lacuna* were rapidly moving. The response pattern in *Patiria*-SCW was very different: 6 snails were moving within the first minute, but none of these were reacting with shell rotation or tentacle waving; at 3 min, 12 were moving and 2 of these were reacting with very mild shell or tentacle movement; at 5 min, 12 were still moving but none of these were reacting.

The most frequent response of specimens of *Lacuna* to actual contact with *Leptasterias* was different from and more intense than the snails' response to the distant seastar or to seastar-scented water. Upon actual contact with *Leptasterias,* most of the snails would rear up on the posterior portion of the foot, raising their

TABLE II

Responses of Lacuna marmorata *to contact with seastars or a control probe in the laboratory and in the field. Two kinds of positive response were seen: rotating the shell (and, concomitantly, waving the cephalic tentacles) and falling off the* Phyllospadix *blade.*

Stimulus	Number of snails (laboratory conditions)			Number of snails (field conditions)		
	Rotating	Falling	Not reacting	Rotating	Falling	Not reacting
Control	0	0	20	0	1	39
Patiria	1	0	19	5	4	31
Leptasterias	6	14	0	6	68	1

cephalic tentacles as well, and fall off the *Phyllospadix* blade away from the point of contact. In the field, fallen snails often would hang by a mucous thread until they contacted a new blade of surfgrass. Then, they would attach to the new blade and move rapidly away while waving the cephalic tentacles and rotating the shell. Although falling was the most common response triggered by contact with the predator, some snails responded with a shell-rotation and cephalic tentacle response similar to that given when the predator was at a distance.

The responses of *Lacuna* to contact with a blunt probe, *Patiria,* and *Leptasterias* are quantified in Table II. Experiments were performed in the laboratory and in the field. All 20 snails tested in the laboratory responded to a light touch with a blunt probe by withdrawing briefly and then by resuming normal activity (*i.e.,* no reaction). No shell-rotation or falling responses were elicited. Similarly in the field, 39 of 40 snails showed no reaction to the probe; 1 snail fell from a surfgrass blade. Contact with *Patiria* elicited a more vigorous response by some of the snails. One snail of 20 in the laboratory responded to *Patiria* with shell rotation; and in the field, 9 of 40 responded in some fashion (5 with shell rotation and 4 by falling). In contrast, almost all of the snails responded vigorously upon contact with *Leptasterias.* All of the 20 snails in the laboratory responded (14 by falling); and in the field, 74 of 75 snails responded (68 by falling). The proportion of snails responding to *Leptasterias* in the field (99%) was far greater than the proportion responding to *Patiria* (22%).

Lacuna, then, has two sets of defensive responses, and the distance between the snail and the predator determines which set will be triggered. As a distant *Leptasterias* approaches, the snail becomes alerted and begins to explore with its cephalic tentacles. At the same time, the snail begins to rotate its shell back and forth through an arc of 360°. This response may lessen the ability of the seastar's tube feet to attach to the shell should contact occur, and it also exposes more of the sensory area of the snail's foot. The general activity level of the snail increases, and some individuals may avoid the approaching predator by moving away. If physical contact is actually made with the seastar, the snail tries to escape by falling off the blade.

Defensive responses of Alia carinata

The responses of *Alia carinata* to *Leptasterias*-SCW were not dramatic. There was no twisting of the body nor waving of the cephalic tentacles. There did seem to be a slight increase in general activity over control levels, but we do not have quantitative data to support this impression. There was, however, one clear

TABLE III

Responses of Alia carinata *to contact with seastars or a control probe in the laboratory and in the field. All positive reactions (rearing, running, falling, and biting) are combined.*

Stimulus	Number of snails (laboratory conditions)		Number of snails (field conditions)	
	Reacting	Not reacting	Reacting	Not reacting
Control	0	20	2	30
Patiria	0	20	1	29
Leptasterias	20	0	23	8

indication that *Alia* was detecting the presence of *Leptasterias*-SCW. When snails were placed in *Leptasterias*-SCW, the tip of the snails' siphon flared almost immediately, and did so in all 20 trials. Furthermore, when *Leptasterias* was actually present nearby, *Alia* often pointed its siphon toward the seastar. Although flaring of the siphon is not itself a defensive response, it indicates that the snail is detecting the presence of the predatory searstar. No siphon flaring occurred in 20 trials with *Patiria*-SCW or with control water.

Definite defensive responses were demonstrated by most specimens of *Alia* upon contact with *Leptasterias* in the labortory and in the field (Table III), but specifics of the responses varied between individuals. In the laboratory, contacts with the snail's siphon most often resulted in *Alia* rearing up on the posterior portion of its foot. With its anterior end thus elevated and with the posterior tip of the foot fixed in place, the body twisted through an arc of 180° away from the point of contact. The snail's pointed shell was then quickly rotated to a position perpendicular to the body, where it remained, exposing the most body tissue (and perhaps coincidentally the sharp operculum) toward the seastar. Snails then ran rapidly away with the siphon flared and pointed toward the seastar. Running in response to *Leptasterias* was accomplished by a muscular, inching movement of the slender foot, in contrast to the smooth, gliding motion characteristic of the snail when it was not reacting.

All 20 snails in the laboratory reacted to *Leptasterias* in some way. In the laboratory, 8 of 20 trials began with the rearing response and concluded with the snail running from the seastar. In the remaining 12 trials the snail reared and began to run, but then either fell off the blade of surfgrass (3 trials) or struck at the seastar with its proboscis (9 trials). These additional responses generally occurred when the seastar continued to contact the snail after the snail had attempted to run. Snails that fell from the *Phyllospadix* blade remained attached by a mucous thread, which they later could climb to return to the blade from which they had fallen. Proboscis strikes were aimed at the tube feet of the attacking seastar with remarkable accuracy. Further, the snail's proboscis, which is as long as its body, was able to bite tube feet approaching from any direction, including the posterior. When bitten by the snail, the individual tube feet of the seastar recoiled immediately, and, if they were attached to the shell, they released their grip. The snail's proboscis would then sweep over the shell surface, as if to check for the presence of remaining tube feet.

The contact responses were given selectively to *Leptasterias* (Table III). In the laboratory, none of 20 snails responded to contact with a blunt probe; and in the field, 2 of the 32 snails tested were scored as having given positive responses when they fell from the plant blade. In the laboratory, none of 20 snails responded to contact with *Patiria:* and in the field, 1 of 30 snails fell from the plant blade. No proboscis strikes were observed against *Patiria.* By contrast, responses were triggered upon contact with *Leptasterias* in a high proportion of trials. In the laboratory, 100% of the snails (N = 20) responded in some fashion; and in the field, 74% (N = 31) responded.

Defensive responses of Notoacmea paleacea

No difference was noted in the behavior of *Notoacmea paleacea* in control water, *Patiria*-SCW, or *Leptasterias*-SCW (20 trials each). In the laboratory, none of the 40 limpets examined in seastar-scented water showed any behavior that could be considered unusual; few (12.5%) moved at all during the 3-min test period. Similarly, field observations on 20 *in situ* limpets indicated that no macroscopic behavioral response was elicited by nearby *Leptasterias.*

Specific responses to contact with *Leptasterias* also were absent. When contacted by a blunt probe in the laboratory (N = 20), the consistent response was to withdraw the soft body parts and to clamp down on the blade for a few seconds. Similarly, when contacted by a tube foot of *Patiria* (N = 20) or *Leptasterias* (N = 20), the body parts were withdrawn and the shell was clamped tightly to the blade. Field observations on 20 *in situ* limpets gave the same result. Neither mushrooming, running, nor twisting of the shell were ever observed after contact. After clamping down, the limpets remained motionless as the seastar crawled over them.

Incorporation of specific chemicals from Phyllospadix *by* Notoacmea paleacea

The curious lack of a behavioral response to *Leptasterias,* coupled with the fact that *Notoacmea paleacea* is known to consume the *Phyllospadix* on which it resides (Test, 1945; Fritchman, 1961; Fishlyn, 1976; Barbour and Radosevich, 1979; Fishlyn, in preparation) suggested that the limpet might be incorporating into its shell or tissues ingested plant chemicals that might act in a form of chemical defense. These chemicals might serve as camouflage, or they might render the limpet distasteful. If *N. paleacea* is incorporating specific chemicals from *Phyllospadix,* then other limpets not feeding on *Phyllospadix* should not contain those chemicals. For comparison, we examined specimens of *Notoacmea insessa,* a stenotopic limpet that feeds on the boa kelp *Egregia menziesii,* a brown alga. We chose *N. insessa* for comparison because it possesses a brown shell as does *N. paleacea,* and it occurs in a similar environment. D. Lindberg (personal communication) states that "*Notoacmea*" *insessa* is not a true *Notoacmea* and properly belongs in another genus.

An alcoholic extract of *Phyllospadix scouleri* blades was a deep, yellow-amber color after treatment with hexane, and a similarly-treated extract of macerated, epizoic-free shells of *N. paleacea* also was yellow in color. Since hexane treatment would have removed the relatively nonpolar pigments (such as chlorophyll and carotenoids), flavonoid pigments, which are relatively polar, were implicated as the most likely source of the color of both extracts. Flavonoids are known to be present in *Phyllospadix* (Nissen and Benson, 1964; S. Brauner, personal communication). In contrast to the yellow color of extracts of *Phyllospadix* and *N. paleacea* shells, alcoholic extracts of the bodies of *N. paleacea* and *N. insessa* were green, and extracts of the shells of *N. insessa* were colorless.

Crude alcoholic extracts (after hexane treatment) of *Phyllospadix* and *N. paleacea* shells were applied as spots to chromatography paper and were easily detected under UV light (254 nm). The spots appeared as dark-absorbing centers surrounded by fluorescing blue-green rings, indicating the presence of phenolic compounds such as flavonoids (Mabry *et al.*, 1970; Harborne, 1973). In contrast, samples of *N. paleacea* bodies, *N. insessa* bodies, and *N. insessa* shells could not be detected on chromatography paper under UV light either before or after fuming with ammonia vapor. The absence of detectable spots after ammonia fuming indicated that phenolic compounds were probably absent from these extracts (Mabry *et al.*, 1970; Harborne, 1973).

Gross spectral features of the crude extracts of *Phyllospadix* and limpet bodies and shells were then examined with a UV spectrophotometer. The extracts of *Phyllospadix* and *N. paleacea* shells absorbed strongly in the 300-nm region and had approximately similar UV spectra. In contrast, extracts of *N. paleacea* bodies and *N. insessa* bodies and shells differed significantly from the spectra of *Phyllospadix* and *N. paleacea* shells. In particular, these extracts did not absorb appreciably in the 300-nm region. Strong absorbing peaks in the 300-nm range are characteristic of flavonoids and simpler phenolic compounds (Harborne, 1973; Harbone *et al.*, 1975; Mabry *et al.*, 1970; Swain, 1976).

The characteristics of the crude extracts suggested that similar phenolic compounds were present in *Phyllospadix* and in the shell of *N. paleacea*. Since these chemicals were not present in detectable amounts in the shell or body of *N. insessa,* incorporation of these plant chemicals into the shell of *N. paleacea* also was suggested.

To further characterize the extracts of *Phyllospadix* blades and *N. paleacea* shells, samples were applied to chromatography paper and developed in two dimensions, first with BAW and then with 15% acetic acid. Two-dimensional chromatography of the extract of *Phyllospadix* produced nine distinct spots detectable under UV light. Four were arrow-shaped, brown, absorbing spots under UV, and each of these turned a more intense yellow-brown when fumed with ammonia vapor. These four spots were colorless in daylight before fuming with ammonia vapor, but all four became a deep yellow after fuming. Two other spots were fluorescent blue under UV. These became a more intense blue after fuming with ammonia vapor. Two more were a fluorescent green under UV, and another was a fluorescent blue-green. The color of all three intensified after fuming with ammonia. All nine spots isolated from the plant extracts are likely to represent either flavonoid pigments (sulphated flavones) or simpler phenolic compounds (*e.g.*, cinnamic acids).

Chromatography of the extract of *N. paleacea* shells produced two distinct spots. Under UV light, one was an arrow-shaped, brown, absorbing spot that turned a more intense yellow-brown after fuming with ammonia. In daylight, this spot was colorless before fuming with ammonia and yellow afterwards. The second spot was a fluorescent blue-green under UV. The color intensified after fuming.

One of the two spots from the extract of *N. paleacea* shells (the brown absorbing spot) closely matched in position, color, shape, and UV spectrum one of the spots from the extract of *Phyllospadix*. The spot from the limpet extract had an R_f value of 0.21 in BAW and 0.75 in acetic acid; the analogous spot from the plant extract had an R_f value of 0.23 in BAW and 0.71 in acetic acid. Before fuming with ammonia, both were colorless in daylight and brown under UV. After fuming, both were yellow in daylight and an intense yellow-brown under UV.

Both spots were arrow-shaped. Finally, the UV spectra of extracts of the two spots were similar. An extract of the limpet spot had absorption peaks at 332 and 269 nm (in 50% ethanol). An extract of the analogous plant spot had peaks at 333 and 275 nm. These data identify both chemicals as flavonoids (probably sulphated flavones), and the close match of physical properties suggests that they differ very slightly, if at all. In addition, the presence of arrow-shaped, dark, absorbing spots with low mobility in BAW and high mobility in aqueous acetic acid approximates properties of sulphated flavonoids found in the eelgrass *Zostera* by Harbone (1975).

The second spot from the extract of *N. paleacea* shells did not have an obvious analog in the extract of *Phyllospadix*. The R_f value of this fluorescent blue-green spot from the limpet extract was 0.35 in BAW and 0.30 in acetic acid. A similarly colored spot was not apparent in this position on chromatograms of plant extract; however, that position on the plant chromatograms was surrounded by three intense, brown, absorbing spots, and these may have masked its presence. Spectral analysis on an extract of the fluorescent blue-green spot indicated a shoulder at 320 nm, another shoulder at 270 nm, and a peak at 242 nm. This spot may represent another flavonoid, or, perhaps more likely, a simpler phenolic acid (*e.g.,* cinnamic acid).

Effectiveness of the defensive adaptations of Lacuna marmorata, Alia carinata, *and* Notoacmea paleacea

The behavioral responses of *Lacuna* and *Alia* are presumed to be defensive adaptations against predation by seastars, and the incorporation of plant chemicals by *N. paleacea* may also function defensively by providing chemical camouflage against predatory seastars. The effectiveness of these adaptations in reducing predation is difficult to assess, but observations on the outcome of encounters between predator and prey provide some information. For instance, encounters involving contact between *Lacuna* and *Leptasterias* were observed on 75 occasions in the field. Eight of these encounters resulted in the capture of the snail; 67 encounters (89%) resulted in the escape of the prey from the predator. If it is assumed that the predator would eat a snail if it could capture it, then one might say that the combined defensive responses of *Lacuna* were 89% effective in reducing predation under the conditions existing in the field site that was studied.

While the defensive responses of *Lacuna* seemed to be fairly effective (*i.e.,* 89% effective), the defensive responses of *Alia* seemed even more effective. Once tube feet of the predator had attached firmly to the shell of *Lacuna,* this snail rarely escaped being eaten. In contrast, the biting response of *Alia* forced the release of any attached tube feet, and sometimes tube feet even were dislodged without the biting response when *Alia* rotated its shell. Often in the laboratory, it was the predator that finally retreated from the prey, and in the field, none of the 31 encounters observed between *Alia* and *Leptasterias* resulted in the capture of the prey. As a further example of the effectiveness of the defensive responses of *Alia,* 4 specimens of *Leptasterias* were maintained in an aquarium with 20 specimens of *Alia* and a small *Phyllospadix* plant for 3 weeks without any of the snails being eaten.

In the *Phyllospadix* beds, the passive defense (*sensu* Ansell, 1969) of *Notoacmea paleacea* also seemed to be more effective in reducing predation than the behavioral defense of *Lacuna.* Twenty encounters between *N. paleacea* and

Leptasterias were observed in the field, and none resulted in the capture of the limpet. A further indication of the relative effectiveness of the defenses of *N. paleacea* and *Lacuna* is found in the diet of *Leptasterias* in the *Phyllospadix* beds.

Over a 2-year period (1976–78), 142 feeding seastars were examined, and most were eating *Lacuna*. In fact, specimens of *Lacuna* comprised 96.2% of the total numerical diet (418 prey total) and 97.1% of the gastropods taken (414 gastropods). Specimens of *N. paleacea* accounted for only 1.4% of the gastropods taken; *Alia* accounted for 0.5%, and two other gastropods, the limpet *Collisella pelta* (juveniles) and the snail *Lirularia succincta*, accounted for 0.7 and 0.2% respectively. Although *Lacuna* certainly is abundant in the *Phyllospadix* beds, this extremely high proportion of *Lacuna* in the diet did not fit our perception of the relative abundance of the three gastropod species in the beds. Consequently, four random samples of *Phyllospadix* from around feeding specimens of *Leptasterias* (575 g total blade wet weight; rhizomes removed before weighing) were taken near the study area in December, 1978. The resident specimens of *Lacuna, N. paleacea,* and *Alia* were counted, and a total of 410 individuals of *Lacuna*, 274 of *N. paleacea*, and 46 of *Alia* were found. The relative abundance of these gastropods on the surfgrass samples (60.1, 33.8, and 6.1% respectively) differed significantly from their relative abundance in the predator's diet (97.1, 1.4, and 0.5% respectively). *Lacuna* was indeed the most abundant prey available to *Leptasterias*, but it was present in the predator's diet in significantly greater numbers than would have been expected if prey were taken at random. Conversely, specimens of *N. paleacea* were significantly underrepresented in the predator's diet. We have interpreted these data on relative abundance as indicating a more effective anti-predator defense by *N. paleacea*. However, it should be mentioned that our data, like other data of this sort, could be interpreted alternately as indicating a preference of the predator for *Lacuna* over *N. paleacea*. Moreover, our population sample (taken once in winter) does not fully complement our feeding observation samples (taken over different seasons spanning two years). Nonetheless, the fact is that the diet of *Leptasterias* seems to be nonrandom with respect to *Lacuna*, *N. paleacea*, and *Alia*. The behavioral cause of this nonrandomness could be prey defenses or predator preferences or a combination of the two.

Discussion

Vigorous defensive responses were given to the predatory seastar *Leptasterias hexactis* by two inhabitants of the *Phyllospadix* beds, *Lacuna marmorata* and *Alia carinata*. Both of these gastropods are capable of sensing the predator from a distance by means of waterborne chemicals, and also upon contact. Furthermore, these defensive responses are given relatively specifically in that they tend not be given to *Patiria miniata*, a seastar that is not a predator on mollusks. Species specificity was not complete, however, because *Patiria* did trigger a small percentage of weak responses. A similar low proportion of weak defensive responses to *Patiria* also was reported for the gastropods *Olivella biplicata* and *Collisella limatula* (Phillips, 1976, 1977).

The response of the mesogastropod *Lacuna marmorata* to distant *Leptasterias* involved rotation of the shell, tentacle waving, and increased general activity. All of these are common components of defensive responses of other gastropods, primarily of archeogastropods (Feder, 1963; Montgomery, 1967; Szal, 1970; Hoffman and Weldon, 1978). One difference, however, is that rotation of the shell previously has been associated most often with responses to contact with the predator.

In contrast, *Lacuna* begins rotating its shell when the predator is still some distance away, presumably in preparation for anticipated contact. Then, upon contact with *Leptasterias, Lacuna* reacts by falling away from the seastar, often escaping into the heterogeneous "jungle-like" mat of surfgrass blades. Falling as a defensive response also has been reported by Dayton, Rosenthal, Mahen, and Antezana (1977) who reported taxonomically various prey falling from, or being swept from, rocks as part of their response to the predatory seastar *Meyenaster gelatinosus.* These authors considered falling to be an effective means of escape from this seastar, and they noted that the degree of effectiveness was increased by conditions of strong surge. As the name surfgrass suggests, *Phyllospadix* flourishes in relatively high-energy environments, and while we have not observed wave surge to aid *Lacuna* in its escape from *Leptasterias,* some benefit may be gained from this by *Lacuna* at high tide. It also should be noted, however, that falling may be hazardous, as the direction and depth of the fall cannot be precisely controlled by the snail. Thus, for example, one of us (DAF) once watched a specimen of *Lacuna* fall away from a specimen of *Leptasterias* only to land on the oral tentacles of the sea anemone *Anthopleura xanthogrammica* and be engulfed.

Our subjective characterization of these defensive responses of *Lacuna marmorata* is that they are vigorous and dramatic. This conflicts, however, with the one previous mention of defensive responses by *Lacuna.* Menge (1972), in a study of the feeding biology of *Leptasterias hexactis,* noted that a defensive response was given by *Lacuna* sp. to this seastar. The response was not described in detail, but it was characterized as weak, while responses of several other gastropods familiar to us were characterized as strong or very strong. There are several possible explanations for the differences between our results and those of Menge (1972): different experimental conditions were used (*e.g.,* in our experiments the snails were attached to blades of *Phyllospadix*), different species of *Lacuna* may have been examined, and different species of *Leptasterias* may have served as the stimulus. This latter possibility is particularly noteworthy since it pertains to results with other gastropods as well. Chia (1966) has synonymized *Leptasterias hexactis* and *Leptasterias aequalis* in Washington State based on specimens from the San Juan Islands, Washington, and this synonymy was extended to species of *Leptasterias* in California (Sutton, 1975) without published evidence (J. T. Carlton, personal communication). There is a distinct possibility that the *Leptasterias* that occurs on *Phyllospadix* in central California is a different species from the *Leptasterias* studied by Menge that occurs on rocks in Washington.

In contrast to the flight responses of *Lacuna* and most other gastropods, the response of *Alia* to *Leptasterias* is much more aggressive. Although *Alia* may flee from *Leptasterias* upon initial contact, if contact with the seastar persists, the snail quickly becomes combative and strikes at the seastar with its proboscis. Often after a series of such defensive strikes, it is the predator that withdraws. This sort of defensive counter-attack has been reported relatively infrequently in mollusks, but Pratt (1974) has reported that the mesogastropod *Crepidula fornicata* jabs with its radula when attacked by the oyster-drill *Urosalpinx cinerea.* Although the proboscis strike of *Alia* is elicited relatively specificially in that it is triggered by *Leptasterias* but not by *Patiria,* we have also seen *Alia* strike at other *Alia* and occasionally at *Lacuna.*

Whereas *Lacuna* and *Alia* give vigorous defensive responses to *Leptasterias. Notoacmea paleacea* gives no detectable flight response to the presence of this predator, in striking contrast to the mushrooming flight behavior of many other limpets (Bullock, 1953; Feder, 1963; Feder, 1972). Yet, very few specimens of *N. paleacea* are consumed by the seastar. We suggest that *N. paleacea* has defenses that are entirely different from those of the other two gastropods. Specifically, we suggest that particular chemicals, flavonoids, present in *Phyllospadix* are incorporated by the limpet and serve as a chemical defense against predation, undoubtedly aided by the tight fit of the limpet's shell with the *Phyllospadix* blade. Flavonoids such as are found in *Phyllospadix* and in *N. paleacea* are thought not to be synthesized by animals, and their rare occurrence in animals usually can be traced to a plant in the animal's diet (reviews by Fox, 1976; Harborne, 1967). Incorporation of flavonoids by marine mollusks has not been reported previously, but a pigment thought to be a flavonoid has been reported in digestive gland, mantle tissue, and other organs of the terrestrial gastropod *Helix pomatia* (Kubista, 1950). The possible occurrence of flavones in the shell of *Cepaea nemoralis, Helix aspersa*, and *H. pomatia* also has been reported (Comfort, 1951). Although the occurrence of plant chemicals in the bodies of terrestrial herbivorous insects is a well-documented phenomenon (flavonoids: Morris and Thomson, 1963; Morris and Thomson, 1964; Harborne, 1967; cardiac glycosides; Rothschild, 1972; Rothschild and Reichstein, 1976; Brower, Brower, and Corvino, 1967; general reviews: Eisner, 1970; Whittaker and Feeny, 1971), we believe that this is the first reported parallel case in the ocean of flavonoids being incorporated by a monophagous, angiosperm-consuming, marine invertebrate.

The presence of specific plant chemicals such as flavonoids in *N. paleacea* could reduce predation in two ways. They might render the limpet distasteful, or they might chemically camouflage the limpet. Only the shell of the limpet contained appreciable amounts of flavonoid, however; the part of the animal that would be consumed by the seastar did not contain appreciable amounts. Location of the incorporated plant chemical(s) in the shell suggests that chemical camouflage may be the more likely possibility. Two additional sets of observations further support this contention. First, when a moving specimen of *Leptasterias* encounters a specimen of *N. paleacea* on a *Phyllospadix* blade, the seastar usually continues without pause to crawl over the limpet. The seastar does not recoil from the limpet, nor does it begin to attack it. The seastar simply seems not to have detected the limpet. Second, two red algae, *Melobesia mediocris* and *Smithora naiadum*, which are regarded by algologists to be epiphytes restricted to *Phyllospadix* and the eelgrass *Zostera* (Abbott and Hollenberg, 1976), also occur on the shells of *N. paleacea*. Since these algae presumably settle at a site based on specific cues identifying that site as *Phyllospadix* (or *Zostera*), settlement of these algae on shells of *N. paleacea* may indicate that the limpet's shells were identified as *Phyllospadix* by the settling algae. A similar misidentification by *Leptasterias* clearly would reduce predation on *N. paleacea*. Finally, the hypothesis of chemical camouflage in the limpet's shell also suggests a function for the one behavioral response that *N. paleacea* does give to contact with *Leptasterias*. Upon contact, the limpet withdraws its soft, body parts and clamps is shell down firmly, just as it does upon contact with a probe. Since the limpet's shell is shaped to fit the plant blade precisely, all the predator would encounter after initial contact with the limpet would be the chemically cryptic shell and *Phyllospadix*. The chemical crypsis suggested here against a marine, nonvisual predator seems to us to be a natural analog of better-known color camouflaging of terrestrial organisms in response to visual predators.

The defensive strategies of *Alia*, *Lacuna*, and *N. paleacea* differ considerably; however, the strategy of each is appropriate to the life-style and morphology of that particular gastropod. Although all three gastropods occur in *Phyllospadix* beds and all are responding to the same predator, *Leptasterias,* each gastropod is interacting with this predator under a different set of circumstances. *Alia* and *Lacuna* are widely distributed, occurring on low intertidal rocks and algae, as well as on *Phyllospadix*. Thus, these gastropods occur in a variety of habitats and their behavioral responses are effective in a variety of habitats. The direct and highly effective proboscis strike of *Alia* is a behavioral response that can be employed anywhere. Indeed, it seems so effective that *Alia* reacts very little to the seastar until actual contact is made, even though *Alia* is able to detect the predator from a distance. Since *Lacuna* does not have a long proboscis like that of *Alia*, obviously the defensive strategy of *Lacuna* must be different. The response that *Lacuna* gives upon contacting a seastar is to fall. Falling was found to be an effective means of fleeing from *Leptasterias* in *Phyllospadix* beds, and it would seem also to be effective in other habitats where *Lacuna* occurs. Similarly, rotation of the shell by *Lacuna,* which seemed to lessen the likelihood that tube feet would attach to the shell, would work equally well regardless of the substratum. In contrast to *Alia* and *Lacuna*, which are eurytopic species, *N. paleacea* is stenotopic. This limpet occurs only on *Phyllospadix*, and the limpet's defenses, which include a chemically-camouflaged, precisely-fitting shell, are adapted specifically for life on this plant. These defenses seem more appropriate to the limpet's lifestyle than a behavioral defense. Among archeogastropods, the most common sort of behavioral defense is flight. However, since *N. paleacea* is dependent on *Phyllospadix,* it would seem that flight from the plant could be as dangerous as the predator.

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SUMMARY

1. In *Phyllospadix* beds of central California, the gastropods *Lacuna marmorata, Alia carinata,* and *Notoacmea paleacea* occur in abundance with the predatory seastar *Leptasterias hexactis*. All three gastropods have defensive adaptations that reduce the frequency of predation by this seastar.

2. *Lacuna* responds to waterborne chemicals ("scent") emanating from distant *Leptasterias* by rotating the shell through an arc of 360°, waving its cephalic tentacles, and increasing general activity. Upon contact with the predator, *Lacuna* flees by falling off the *Phyllospadix* blade.

3. *Alia* does not respond vigorously to the scent of distant specimens of *Leptasterias*. Upon contact with *Leptasterias, Alia* rears up on the posterior portion of the foot and then begins to run away. If contact with the seastar persists, the snail strikes at the seastar's tube feet with its proboscis.

4. *N. paleacea* does not give a behavioral defensive response to *Leptasterias*. Rather, this stenotopic limpet may be chemically camouflaged, and this camouflage, together with the limpet's precisely fitting shell, may reduce predation by *Leptasterias*. *Notoacmea paleacea* eats *Phyllospadix*, and at least one specific chemical from the plant, a sulphated flavonoid pigment, also is found in the shell of this limpet. Due to the presence of specific, host-plant chemicals in the shell of *N. paleacea*, *Leptasterias* may fail to detect the limpet against the chemical background of the host plant.

5. *Lacuna* is the most frequently consumed prey of *Leptasterias* in the *Phyllospadix* beds, yet 89% of the 75 encounters observed between *Lacuna* and *Leptasterias* in the field resulted in the successful escape of the prey. The defensive adaptations of *Alia* and *N. paleacea* seem to be even more effective, since these gastropods are consumed only rarely by *Leptasterias*.

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EFFECTS OF SALINITY AND TEMPERATURE ON THE PREDATION RATE OF *THAIS HAEMASTOMA* ON *CRASSOSTREA VIRGINICA* SPAT

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Metabolism and activity of aquatic invertebrates are influenced by environmental factors. In the estuarine habitat, two important factors are temperature and salinity (Kinne, 1971). To a great extent, the interaction and fluctuation of these two factors regulate the activity and distribution of estuarine invertebrates. Kinne (1971) has stressed the importance of multifactorial experiments involving the effects of salinity and temperature upon estuarine invertebrates.

Previous studies have been conducted on the southern oyster drill, *Thais haemastoma,* which is a serious predator of the commercial oyster, *Crassostrea virginica.* Aspects of predation and estuarine distribution of *T. haemastoma* have been described (Burkenroad, 1931; St. Amant, 1938, 1957; Butler, 1953; and Belisle and Stickle, 1978). St. Amant (1938, 1957) reported that *T. haemastoma* is unable to penetrate estuaries below salinities of 15‰, although later laboratory work (Stickle and Howey, 1975) indicated that drills can tolerate salinities of 5‰ at 20° C for at least 10 days. Below 12° C, *T. haemastoma* becomes torpid and inactive (St. Amant, 1957).

The effects of temperature and salinity on the rates of predation by other, closely related species, *Urosalpinx cinerea*, *Eupleura caudata* and *Thais lapillus*, have been investigated (Hanks, 1957; Manzi, 1970a, b; Zachary and Haven, 1973; and Bayne and Scullard, 1978). Using *Crassostrea virginica* spat and *Mytilus edulis* as prey, Manzi (1970a) demonstrated that the predation rates of *U. cinerea* and *E. caudata* increased with temperature and salinity. Maximum predation rates were observed at the highest temperature and salinity combination used, 26‰ salinity at 25° C. Zachary and Haven (1973) found the activity of *U. cinerea* to increase with salinity. However, drills exposed to fluctuating salinity regimes exhibited lower levels of activity and survival than those under constant salinity. In these trials, the magnitude of the salinity fluctuations was an important factor affecting drill activity and survival (Zachary and Haven, 1973). The predation rate of *Thais lapillus,* preying on *M. edulis,* is temperature-sensitive and shows seasonal variability (Bayne and Scullard, 1978).

The purpose of this investigation was to determine the effect of temperature and salinity interactions on the predation rate of *T. haemastoma* on spat of *C. virginica.* To more accurately reflect the estuarine conditions to which drills are frequently exposed, the effects of simulated tidal fluctuations of salinity on the predation rate of the oyster drill were also studied.

MATERIALS AND METHODS

Oyster drills

Specimens of *T. haemastoma* were collected during the summer and fall of 1977, and early spring of 1978, from the vicinity of Caminada Pass near Grand Isle, Louisiana, U. S. A. Drills were placed in 38-liter aquaria at room temperature (22°–25° C) and field salinity (Instant Ocean sea water mix). Salinity was adjusted 2‰ per day until the desired salinity was attained. The drills were then allowed to acclimate at constant salinity for at least one week prior to temperature adjustment. Snails were transferred from room temperature, and the water temperature was allowed to change gradually to the experimental temperature over a period of about 8 hr. Drills were acclimated for at least 10 days at constant temperature and salinity prior to the start of the experiment. For 20° and 30° C experiments, the aquaria were placed in a large temperature-controlled water bath. An environmental chamber was used for the 10° C and low-temperature threshold experiments. The drills were under normal light intensity from ceiling illumination and there did not appear to be any phototactic response by the drills.

Oyster spat

Cultured, cultchless *C. virginica* spat were used as prey. Spat averaged approximately 2 to 3 cm in length and 0.8 to 1.0 g total weight. Spat size and weight varied with season, but were uniform within shipments. During the summer of 1977, spat were supplied by the oyster hatchery operated by the Gulf Coast Research Laboratory at Ocean Springs, Mississippi. The Horn Point Environmental Laboratory operated by the University of Maryland at Cambridge, Maryland, supplied spat during the fall of 1977 and summer of 1978. Upon arrival, the spat were placed in holding aquaria at room temperature and at constant salinities of 15‰ and 25‰. Spat at 15‰ were directly transferred to 7.5, 10.0, 15.0 and 20‰ experimental aquaria and spat at 25‰ were transferred to 20, 30, and 35‰ experimental aquaria. The use of cultured, cultchless oyster spat insured uniform prey size and equal caloric value for each prey item consumed. Dame (1972) found no significant seasonal variation in the caloric content of small (<20 g whole weight) intertidal oysters.

Feeding rate determinations

To determine the individual feeding rates, a drill was placed in each of 8 compartments formed by dividing 38-liter aquaria with slotted plexiglass panels. Oyster drills of uniform length (~45 mm) were selected to remove size as a variable within experimental design. The feeding rate was determined by removing and counting the number of spat consumed each day. A density of 5 spat per drill was maintained throughout the experiment. By weighing the spat beforehand and the empty valves afterwards the total wet weight of oyster flesh consumed over the course of the experiment was determined. Per cent body water was determined by exposing a group of spat to each temperature and salinity combination. Per cent body water was measured as: (total wet wt. minus total dry wt.) times 100, divided by the total wet wt. The per cent body water was used to convert total wet weight of oyster flesh consumed to a dry weight basis. The predation rate was measured as the average daily feeding rate (ADFR, spat per drill/day) and the ingestion rate as grams dry weight (DW) consumed per drill during the 21-day experiment. The initial and final length and weight, and the sex of each drill were recorded.

The drills were provided with adult specimens of *C. virginica* during acclimation until 1 week prior to the start of the experiment. At that time the drills were provided with oyster spat. Following acclimation and conditioning, the predation rate was measured daily for 21 days.

Salinity and temperature regimes

Feeding rates were determined at steady state salinities of 7.5, 10, 15, 20, 25, 30 and 35‰ at 10°, 20° and 30° C. The 20° C experiment was performed in August, 1977, the 10° C experiment in November, 1977, and the 30° C experiment in July, 1978. The 35‰ treatment at 20° C was omitted.

Oyster drills were exposed to simulated diurnal salinity fluctuation patterns of 30 to 10 to 30‰ and 10 to 30 to 10‰ at 10°, 20° and 30° C, using procedures similar to those given by Stickle and Ahokas (1974). During diurnal cycles, salinity was increased or decreased over a 10-hr period, held at slack water for 2 hr, changed in the opposite direction over 10 hr and held at the initial salinity for 2 hr. These cycles ran continuously for 21 days. Each salinity fluctuation regime was duplicated.

Temperature threshold and group versus individual experiments

The low temperature threshold at which drills begin to prey on oyster spat was evaluated. The predation rate of drills at salinities of 20‰ was determined for temperatures of 12.5°, 15.0° and 17.5° C.

Finally, individual feeding rates were compared with those of a group at 25° C and 20‰. This experiment was carried out in order to test the hypothesis that drills that are more efficient at killing oysters may provide food for scavenging conspecifics. Because oyster spat were too small to permit aggregate feeding, adult oysters were used in this experiment. Both the temperature threshold and the group *vs.* individual predation rate experiments were performed in July, 1978.

Statistical analyses

Two-way analysis of variance was performed using the general linear model method on the data from the 20° and 30° C experiments (Statistical Analysis System, Barr, Goodnight, Sall and Helwig, 1976). The two variables analyzed were dry weight of oyster flesh consumed during the trial and the average daily feeding rate. The general linear model included salinity, temperature and sex, plus all four possible interactions and was corrected for drill length and weight differences in each cell. Duncan's multiple range test was used to determine significant differences of means of the two variables within each temperature treatment. This test was also used to compare means in the low temperature threshold experiment. A t-test was used for the group versus individual feeding rate trial. Statistical significance is given at the $P < 0.05$ level.

RESULTS

The average daily feeding rate (ADFR) and dry weight of oyster flesh consumed (DW) were highly correlated in all experiments ($r = 0.925$). These two variables are equal indicators of predation.

Statistical analysis of the data for the temperature threshold, and the 20° and 30° C experiments do not indicate significant ($P > 0.05$) relationships between predation rate and drill total weight or length. Likewise, 2-way analysis of vari-

TABLE I

Results of the temperature threshold experiment. ADFR = average daily feeding rate; DW = g dry weight of oyster flesh consumed in 21 days. Values are means ± standard error. Salinity was 20‰; N = 8 for each temperature.

Temperature (°C)	ADFR	DW
12.5	0.119 ± 0.018	0.254 ± 0.049
15.0	0.875 ± 0.111	1.625 ± 0.216
17.5	1.387 ± 0.086	2.341 ± 0.123

ance for the 20° and 30° C experiments reveals no significant effects related to sex. Therefore, both sexes were combined in the statistical model.

Mortality and egg capsule deposition

Mortality during the period of acclimation for the 20° and 30° C trials, the temperature-dependent feeding threshold experiment, and the group versus individual feeding rate trial was less than five per cent. No mortality was observed during any of the experimental periods in these experiments. In the 10° C trial, 7 out of 78 snails died during the last 2 weeks of the experiment. Three drills died at both 30 and 35‰ and one drill died in the 30 to 10 to 30‰ cycle. These drills were not replaced.

Egg capsule deposition was observed among the snails collected in the early spring of 1978. These snails were used for the 30° C trial, the temperature threshold experiment and the group vs. individual feeding experiments. Prolific capsule deposition lasted approximately 6 weeks during the period of acclimation at the constant acclimation salinities of 15, 20 and 25‰ at 30° C and 20‰ at room temperature (approximately 25° C). No capsule deposition occurred during the 21-day experimental period.

Temperature and salinity interactions

The temperature threshold for predation was between 10° and 12.5° C and a temperature-dependent relationship of predation rate exists between 12.5° and 17.5° C (Table I). Predation and ingestion rates in the temperature threshold experiment were significantly different at each temperature as revealed by Duncan's multiple range test.

Although sporadic predation was observed at 10° C, the predation rate was effectively 0 (Fig. 1). During the 21-day trial; 2 spat were consumed by 8 drills at 15‰, 3 spat at 20‰ and 1 spat at 25‰ and the 10 to 30 to 10‰ cycle. No spat were consumed in the other salinity treatments. The drills remained attached to the substrate during the experiment, but were in a "torpid" state and showed very little movement.

Duncan's multiple range tests performed on predation and ingestion rate means at 20° C show that both ADFR and DW were significantly greater for drills at salinities of 15‰ than for drills at 7.5‰ and 10 to 30 to 10‰ (Fig. 1). At 7.5‰, the predation rate was significantly less than those observed between 10 and 25‰. Predation rates at 10, 15, 20, 25 and 30‰ and in the 2 fluctuating cycles were not significantly different.

At 30° C, the 7.5‰ ADFR was significantly less than ADFRs for all other

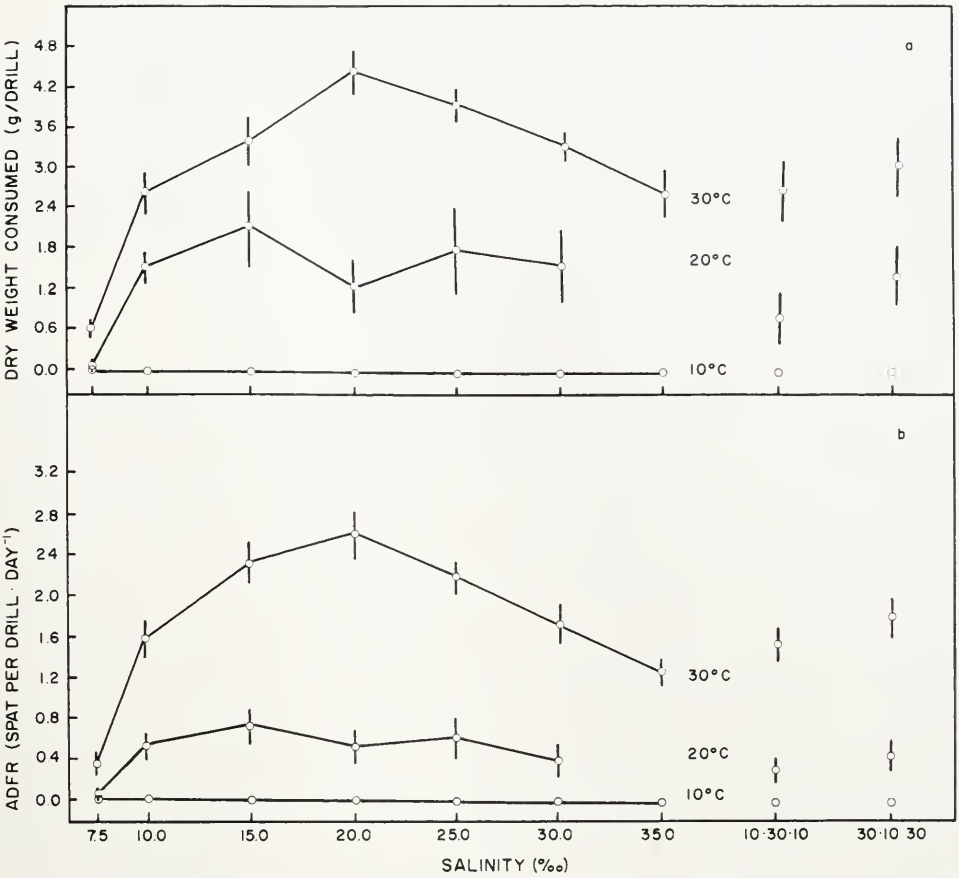

FIGURE 1. Ingestion rate of *Thais haemastoma*, expressed as g dry weight of oyster flesh consumed during 21 days, a, and predation rates as the average daily feeding rate, b. The ingestion and predation rates are presented as means (symbol) ± 1 standard error (vertical bar).

salinities, while the 20‰ ADFR was significantly greater than the predation rates at all other salinities (Fig. 1). The predation rates at salinities of 15 and 25‰ were significantly greater than the ADFRs for 10, 30 and 35‰ and the two fluctuating salinity cycles. The predation rates in the latter five salinity treatments were not significantly different. The means for ingestion rate reveal slightly different results than the ADFR. Dry weight consumption at 20‰ was significantly greater than at all other salinities except 25‰. Dry weight consumption at 35‰ was significantly less than consumption at 30‰ and during the 30 to 10 to 30‰ fluctuating salinity cycle.

Overall, there was a significant interaction of salinity and temperature effects upon predation rates. The effect of constant salinity on predation rates at 20° C was not as pronounced as at 30° C (Fig. 1). There was no change in predation or ingestion rates above 20‰ at 20° C, while there was a significant decrease in predation rates above 20‰ at 30° C. However, there was no significant interaction between salinity cycle (*i.e.* 10 to 30 to 10‰ *vs.* 30 to 10 to 30‰) and temperature effects upon predation rates.

Group vs. individual predation rates

During the 17 days of this experiment, drills consumed 20 large oysters in the divided aquarium and 21 oysters in the undivided, or group, aquarium. The total consumption of oysters by snails in the divided aquarium was 10.5 ± 1.0 g dry weight per drill and 11.7 ± 0.1 g dry weight in the undivided aquarium. No significant difference in consumption of oyster flesh existed between the two groups. The predation rate for the group was 0.13 oysters/(drill·day) while the individual drills consumed 0.12 oysters/(drill·day). Large oyster entire weight averaged 234 g.

DISCUSSION

Predation by *Thais haemastoma* upon oyster spat is affected by salinity and temperature. The effect of constant salinity treatments on the predation rate differs with temperature. At 20° C consant salinity treatments between 10 and 30‰ did not influence predation rates. At 30° C, there is a narrow optimum for predation at 20‰, above which the predation rates decrease. Manzi (1970a) also reported that temperature and salinity interact, affecting the predation rates of *Urosalpinx cinerea* and *Eupleura caudata*.

Previous studies have shown that the response of estuarine organisms to chronic salinity stress or acute salinity stress (direct transfer experiments) are different from natural tidal fluctuations of salinity (Stickle and Ahokas, 1974; Stickle and Howey, 1975; Findley, Belisle, and Stickle, 1978). Predation rates of *T. haemastoma* in 10 to 30 to 10‰ and 30 to 10 to 30‰ fluctuating salinity cycles are not significantly different. Therefore, the initial acclimation to constant salinity prior to starting the salinity cycles does not affect the predation rates. Furthermore, the predation rates in these fluctuating salinity cycles are not significantly different from those in the constant acclimation salinities of 10 and 30‰ at each respective temperature. This is evidence of functional euryhalinity in *T. haemastoma*. Tidal fluctuations of this magnitude occur infrequently and are considered near the maximum naturally occurring salinity fluctuations, yet are compatible with *T. haemastoma* feeding and survival.

The low temperature limit for predation in *T. haemastoma* from the Gulf of Mexico is slightly higher than that for a closely related species from the North Atlantic coast, *Urosalpinx cinerea*. Specimens of *U. cinerea* collected from Long Island Sound began feeding between 5° and 10° C at 25‰ (Hanks, 1957). The feeding rate increased until the temperature reached 25° C and then declined at 30° C. *T. haemastoma* fed sporadically below 12.5° C and at its maximum rate at 30° C, the upper temperature investigated in this experiment. These differences in response to temperature are thought to be a result of the geographic distribution of the two species. *T. haemastoma* has a more southerly distribution, and is therefore exposed to higher temperatures than *U. cinerea*.

Thais haemastoma also displayed a greater tolerance to salinity stress than *Urosalpinx cinerea* and *Eupleura caudata*. The minimum salinity compatible with predation by the latter two species was 12.5‰ at 15°, 20° and 25° C (Manzi, 1970a). In this study, *T. haemastoma* fed lightly at 7.5‰ and 20° and 30° C. In a preliminary study to determine lower lethal limits, adult specimens of *T. haemastoma* tolerated 5‰ at room temperature (approximately 25° C) for 1 month without mortality, although the snails appeared swollen and did not feed.

These results indicate that *T. haemastoma* is a euryhaline organism with extreme tolerance to salinity stress. Previous published data have demonstrated this species to be an osmoconformer and to physiologically respond to changes in salinity in a manner similar to stenohaline organisms. Oxygen consumption in *T. haemastoma* decreases when the salinity is fluctuated upward or downward from the acclimation salinity, a characteristic of stenohaline organisms (Findley *et al.*, 1978). This species also displays little ion or volume regulation. In fluctuating salinities, hemolymph composition and osmolality track ambient sea water (Stickle and Howey, 1975).

Although *T. haemastoma* can prey on oyster spat over a wide range of temperatures and salinities, an understanding of its energy budget balance is lacking. However, the energy budget for a related muricid gastropod, *Thais lapillus,* varies as a function of temperature and season (Bayne and Scullard, 1978). The "scope for growth," the number of calories remaining after the metabolic demands of the individual were met, was greater for specimens of *T. lapillus* collected in the fall (16° C) than for drills collected in the spring (9° C). Since prey selection was assumed not to vary seasonally, the difference was primarily due to changes in predation rate and oxygen consumption (Bayne and Scullard, 1978). The estuarine environment is typified by such seasonal variations in salinity and temperature, similar to those explored in this experiment with *T. haemastoma.* The predation rate and oxygen consumption of the southern oyster drill varies with temperature and salinity and future experiments are planned to determine if the scope for growth also changes for this species.

Field observations indicate that *T. haemastoma* is rarely found in estuarine regions where the average salinity is less than 15‰ (St. Amant, 1938). If adult drills are tolerant of salinity stress, why are they excluded from estuarine regions of less than 15‰? There is limited evidence for speculating that reproduction and larval development are the critical points of the life cycle that are sensitive to reduced salinity. Capsule deposition in the laboratory at 30° C did not occur below 15‰ and Wells (1961) reported that larval specimens of *T. haemastoma* did not survive the 5-day test period below 10‰. It appears that if some larvae did invade the upper limit of an estuary during low river discharge (elevated salinity) and mature, they would fail to form a breeding population. Exposed to chronic low salinities, less than 15‰, their predation rates would be greatly reduced. Isolated populations would be difficult to detect because *T. haemastoma* does not always bore through oyster valves, therefore they leave little permanent evidence of predation (McGraw and Gunter, 1972).

In the laboratory, specimens of *T. haemastoma* characteristically feed in aggregates on adult specimens of *C. virginica.* Although it is possible that group feeding by drills would exceed the total rate for individual drills, this was not the case in our study. Individual snails, averaging 52.6 mm in length, were as capable of killing and feeding on adult oysters as members of a feeding aggregation.

T. haemastoma possesses a paralytic venom that causes oysters to gape, exposing the oyster tissue. In specimens of *T. haemastoma* from Italy, Roseghini (1971) found dihydromurexine from hypobranchial gland extracts. Whittaker (1960) found senecioylcholine in the hypobranchial gland of *T. floridana* (= *T. h. floridana*) from Florida. Both of these compounds function as powerful neuromuscular blocking agents. This paralytic capability may partially explain why *T. haemastoma* is able to prey over a wide range of salinities. No preference for drilling or envenomation was noted at any salinity or temperature during this experiment. However, the use of fairly small spat made such a determination difficult.

The energy expenditures required for either method of attack are totally unknown, but *T. haemastoma* has an advantage in possessing a typical muricid radular apparatus and accessory boring organ (See Carriker, 1978) as well as a venom.

Because *T. haemastoma* tolerates ambient salinity well below its distributional limit in estuaries and it also maintains high predation rates below the 15‰ limit suggested by field observations (St. Amant, 1938, 1957), other physiological factors may cause stress in the southern oyster drill. Indexes of physiological stress have recently been developed with other molluscs which may aid in explaining the disparity between the field distribution of the species and laboratory observations of its metabolism and activity as related to salinity. These indexes include scope for growth, growth efficiency and oxygen : nitrogen (O : N) ratios (Bayne and Scullard, 1978; Widdows, 1978). These stress indexes are currently being applied to *T. haemastoma* as a function of temperature and salinity.

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SUMMARY

1. The predation and ingestion rate of *Thais haemastoma* on oyster spat is sensitive to temperature and salinity. The effect of salinity on predation rate was different at 20° and 30° C.

2. The temperature threshold for predation was between 10° and 12.5° C; drills were not observed to feed below 7.5‰.

3. Drills exposed to diurnal tidal fluctuations of salinity, reflecting the estuarine environment, had predation rates significantly less than those at the optimal constant salinity (20‰) at 30° C, but were not significantly less at 20° C. The predation and ingestion rates in the fluctuating salinity cycles were not significantly different than rates for drills at the constant acclimation salinities of 10 and 30‰.

4. Predation rates were not significantly affected by drill sex or weight and length. However, the experiments were designed to exclude size effects by selecting drills within a size range of 45 to 55 mm.

5. Predation rates were not significantly different between large drills feeding as individuals (1 drill/adult oyster) or as aggregates (>1 drill/adult oyster).

6. The predation and ingestion rate data indicate a functional euryhalinity in *T. haemastoma.* This species of oyster drill can tolerate changes in its physiology accompanying changes in temperature and salinity and still maintain its ability to function as an efficient predator.

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MECHANICAL PROPERTIES AND LOCOMOTOR FUNCTIONS OF EEL SKIN

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The functions of fish skin are numerous, and are generally categorized as forms of protection from or adjustment to the environment. Eel skin in particular performs these functions admirably. The fact that tanned eel skin has been used as door hinges in Scandinavia (Knut Schmidt-Nielson, personal communication) attests to its toughness. With its ability to produce copious quantities of mucus the skin can also assist the animal in adjusting to rather extreme environmental conditions—eels are not only euryhaline but are also capable of aerial cutaneous respiration (Berg and Steen, 1965).

While performing these functions the skin must also provide both a rigid framework to support the body contents and a flexible covering to allow whatever changes in shape are necessary for locomotion. A system of collagen fibers in the skin of fishes arranged in alternating layers forming right and left helices about the long axis of the animal allows for shape changes. This highly ordered arrangement has been described for elasmobranchs (Motta, 1977; Wainwright, Vosburgh & Hebrank, 1978) and teleosts (Fujii, 1968; Nadol, Gibbons and Porter, 1969; Brown and Wellings, 1970; Hawkes, 1974; Smith, 1956; Willemse, 1972; Videler, 1975).

Engineers will recognize this design feature; they have for some time been reinforcing thin-walled pressurized cylinders by winding them with inextensible fibers. Such a design creates a lightweight cylinder wall which is strong against explosion and has the additional advantage of permitting extensions in any direction except those of the fibers themselves, *i.e.*, they resist torsion of the cylinder. In the directions of the circumference and long axis of the cylinder the greatest range of extension is permitted, these are the directions which bisect the angles between a pair of crossed-helices. The angle that these helices make with the long axis of the cylinder is called the fiber angle. For a range of fiber angles excluding those very near 0° and 90°, the cylinder can be bent without kinking or wrinkling.

The convex side of a bent cylinder is stretched in the longitudinal direction and compressed in the circumferential direction; on this side the fiber angle decreases. The reverse occurs on the opposite, or concave, side. In this way the cylinder, or a fish, can undergo a range of movements without changing its body form or volume (Clark, 1964), and without kinking or wrinkling.

With their study of shark skin, Wainwright *et al.* (1978) provide the first demonstration of the importance of a crossed-fibrillar array of collagen to fish locomotion. These authors found the skin acts as an external tendon, transmitting force and displacement in parallel with the attached, underlying muscles. Because a force applied to the skin generates a greater bending moment than the same force applied near the backbone, the mechanical advantage of the axial musculature is enhanced by skin capable of transmitting forces down the length of the fish.

Like the shark, the eel swims by undulatory propulsion, but differences in body form between the two are apparent. The shark is constructed with the bulk of its musculature far anterior to its primary propulsive surface—the caudal fin. The eel has its musculature distributed more evenly along its length, and uses nearly this whole length as its propulsive surface—it lacks a distinct caudal fin. While it is easy to imagine the benefits an external tendon could provide the shark, *i.e.,* a mechanism for transmitting forces generated by the muscles to the tail, we might wonder whether similarly constructed skin could benefit the eel.

In view of the morphological differences between sharks and eels, the purpose of this study was to investigate the structural features and mechanical properties of eel skin as they relate to the locomotor behavior of the fish.

MATERIALS AND METHODS

Experimental animals. Eleven adult American eels (*Anguilla rostrata*) ranging in length from 41 to 64 cm were used for the mechanical tests and locomotion studies described below. They were obtained live from brackish intracoastal waters of North Carolina from Manteo to Wilmington, and kept in aquaria at native salinity until killed. Skin was removed from the refrigerated carcasses as needed. For histological studies, skin of the adult eels described above was used, as well as that of several elvers, the smallest of which was 20 cm in length. These were obtained by seining in fresh water creeks in North Carolina, and were kept frozen until studied.

Structure of the skin. Cryostat (American Optical, Model 830) sections ten microns thick were made from fresh skin samples, mounted on slides with distilled water, and examined using a Leitz Ortholux polarized light microscope equipped with a first-order red compensator. Other samples were fixed in 10% formalin, sectioned and stained with Verhoeff's elastin stain according to the procedure outlined in Humason (1962). Fresh skin samples were examined using a Wild M-5 dissecting microscope in order to determine the fiber angles. This was accomplished using the protractor stage of the microscope.

Mechanical testing. To test for elasticity in eel skin, several types of stress-strain tests were conducted. Uniaxial tensile stressing tests were performed on skin samples in order to quantify stress-strain relationships in the direction of the collagen fibers and in both the longitudinal and hoop (circumferential) directions. Square or rectangular skin samples measuring several centimeters on a side were cut from the fish between the dorsal and anal fins, or in regions anterior to these, between the mid-dorsal and mid-ventral lines. As much muscle as was possible was removed from the skin using razor blades and scissors. Snap-swivels or alligator clips connected to eighteen-pound test fishing leader wire were attached to the edges of the samples for testing in a stress-strain machine described elsewhere (Wainwright *et al.,* 1978). To test for extension in the actual fibers within the skin, samples were cut and stressed at 45° to the long axis of the animal (the fiber angle determined in prior dissections); during stressing the orthogonal sides of the skin were allowed to deform freely. In a similar manner skin was cut and stressed uniaxially in the hoop and longitudinal directions.

An additional series of stress-strain tests was conducted by applying an increasing tensile load to the longitudinal axis while maintaining the hoop axis at a constant stress. In order to maintain this constant stress the hoop sides were allowed to compress or extend as necessary during the extension and compression of the longitudinal sides. The primary purpose of these biaxial stressing tests was to determine the relative contributions of the crossed-fiber system and the interfibrillar matrix material to the mechanical properties of the whole skin. If the extensibility of the skin is controlled solely by the helical fibers, the ratio of the hoop to longitudinal stresses applied is always equal to the tangent of the fiber angle resulting from these stresses times the tangent of the initial fiber angle. Using this relationship a longitudinal stress-strain curve can be constructed for any constant hoop stress applied, that will predict skin properties if they are due only to a set of continuous fibers. Comparison with the experimentally obtained stress-strain curve for whole skin should allow the role of the interfibrillar matrix to be assessed.

The secondary purpose of this experiment was to test for isotropy in the hoop and longitudinal directions predicted by a 45° fiber angle. To do so the experimental conditions were simply reversed and applied to previously untested samples, so that an increasing tensile stress was applied to the hoop axis while the longitudinal axis was maintained at a constant stress.

Extensions of the skin during swimming. Stress-strain curves for the skin cannot be very meaningful to our understanding of fish locomotion unless we know where the range of *in vivo* stresses and strains falls on the experimentally determined stress-strain curves. For this reason extensions of the skin in the hoop and longitudinal directions were measured in the following way: An eel was anaesthetized by covering it with ice for 2 to 3 hr, then 4 stitches were sewn in the skin on the side of the animal with heavy white thread. The four knots thus made formed a rectangle centered over the lateral line and an imaginary line running dorsoventrally located about three quarters down the eel's length. The eel was then warmed gradually and returned to an aquarium, after which it appeared to behave normally.

The following day the eel was placed in a circulating flow tank and filmed first from the side and then from above, using a Canon Scoopic 16-mm cine camera equipped with a close-up lens. The films obtained were analyzed using a

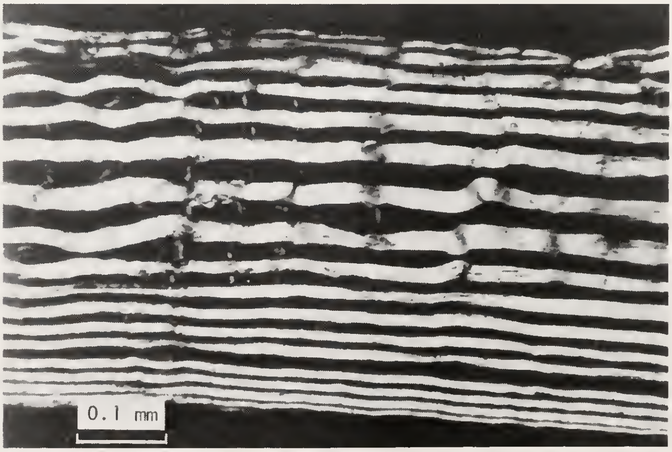

FIGURE 1. Polarized light micrograph of a radial section of eel skin cut parallel to one set of fibers. Seventeen fibers (or fiber layers) are seen in long section and six radial fibers are visible in the left half of the section.

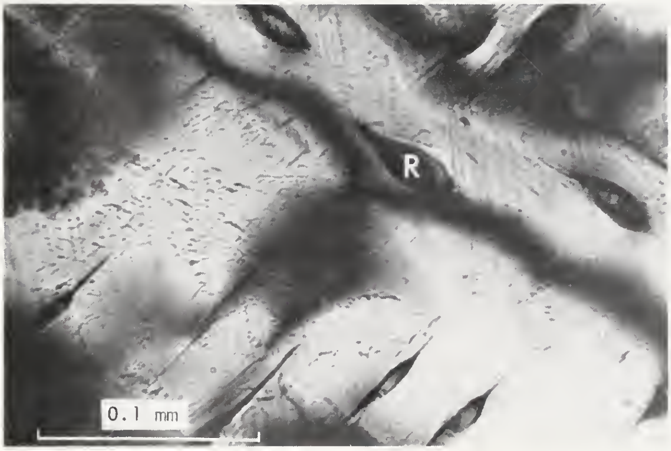

FIGURE 2. Polarized light micrograph of a tangential section of the fibrous region of eel skin. The long axis of the photograph is approximately parallel to the long axis of the fish. R, transverse section of radial fiber.

Perceptoscope; in the analysis the distances between the projected images of the pairs of knots were measured to the nearest 1/50 mm on the fish with vernier calipers.

RESULTS

Structure of the skin. Eel skin differs from the skin of most teleosts in that the cycloid scales are completely embedded in the skin and are more or less isolated from each other (van Oosten, 1957). The pattern of the scale arrangement, however, can be readily observed without dissection due to the pigmentation in the outer layer of the skin which demarks the underlying scales. The oblong scales generally lie with their long axes oriented at an angle of about 45° to the long axis of the animal, but very close to the mid-dorsal line the scale angle decreases to as little as about 10°. The scales can be seen to lean both dorsocranial and dorsocaudal, and these two orientations appear to be distributed randomly, giving the skin a shingled appearance. Surrounding the scales is a gelatinous material, under which lies a thick layer of collagen fibers. Microscopy reveals that alternating sheets of parallel fibers comprise this layer. Figure 1 is a polarized light micrograph of a section cut perpendicular to the skin and parallel to one set of fibers; seventeen fibers (one per layer) can be seen in long section. At the upper and lower boundaries of the dense fibrous layer the fibers are smaller in diameter. Interspersed through the helically-wrapped fibers are radially oriented fibers. These are seen in cross-section in the photomicrograph shown in Figure 2. The diameter of the radial fibers is approximately equal to that of the helical fibers; they are generally round in cross-section but may become distorted where they intersect the helical fiber layers. Beneath the fibrous layer is the subcutis, onto which the underlying muscles insert obliquely.

Fixed sections of eel skin stained negatively with Verhoeff's elastin stain. Counterstaining in Picro-Ponceau indicated that both the helical and radial fibers are probably composed of collagen.

The skin thickness and fiber diameter were found to increase with increasing

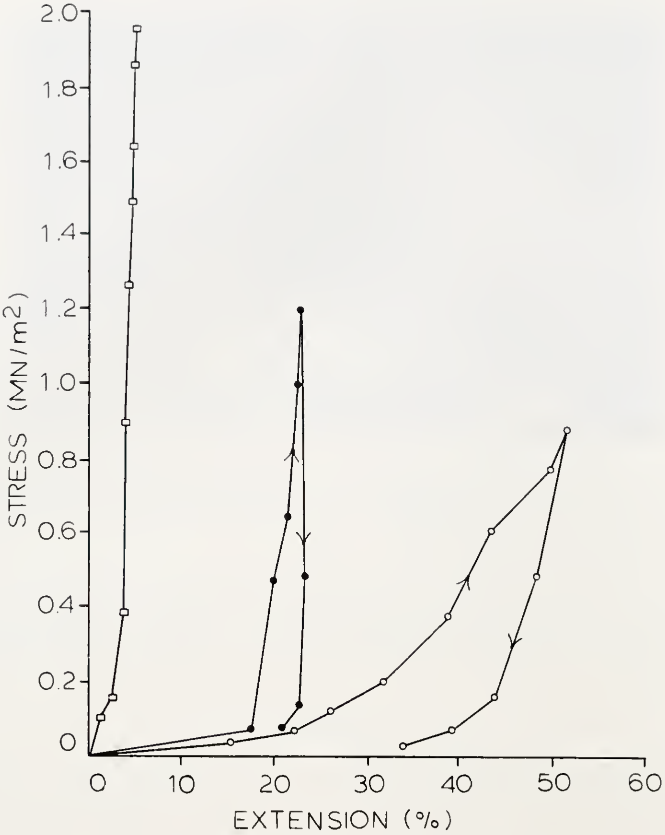

FIGURE 3. Typical stress-strain curves for eel skin stressed uniaxially in three directions. Open box, skin stressed in the direction of one set of fibers; closed circle, skin stressed in the hoop direction; open circle, skin stressed in the longitudinal direction. Loading and unloading curves are indicated by arrows for skin stressed in the hoop and longitudinal directions.

body length. A 20-cm long elver had skin 150 to 180-μm thick and fibers of diameter ranging from about 5 to 10 μm, while a 56-cm adult had skin 500 to 530-μm thick and fibers of about 10 to 25 μm in diameter. The elver's skin contained 11 to 13 pairs of fiber sheets and the adult's had 14 to 16 pairs.

Microscopy reveals that the long axes of the scales indicate the angle of the underlying collagen fibers. This is not true near the mid-dorsal line, however, where the scale angle becomes very low but the fiber angle does not change as the mid-dorsal line is traversed. A fiber angle of about 45° was found to prevail throughout the length of the fish (mean fiber angle = 45.7° ± 4.7, $n = 87$). This angle is the same for both the anterior and posterior leaning fibers, and does not change through the lateral line.

Mechanical testing. In Figure 3 the results of three uniaxial stretching tests are shown in the form of stress-strain curves for skin stretched in the direction of one set of fibers and in the hoop and longitudinal directions. In the direction of the fibers very low extensions are obtained at high stresses. The fibers therefore appear to be reasonably inextensible, especially within the range of stresses applied

TABLE I

Comparison of mean terminal elastic moduli (E) for eel skin stressed uniaxially in three directions

Direction	
On-fiber	$E = 1.56 \times 10^8 N/m^2$ $(n = 10, s.d. = 1.44 \times 10^8)$
Hoop	$E = 1.47 \times 10^7 N/m^2$ $(n = 10, s.d. = 1.28 \times 10^7)$
Longitudinal	$E = 3.54 \times 10^6 N/m^2$ $(n = 10, s.d. = 2.52 \times 10^6)$

to the hoop and longitudinal directions. The elastic modulus obtained as an average of ten on-fiber pulls was 0.16 GN/m^2 (gigaNewtons per square meter), which is slightly lower than the elastic modulus of 0.43 GN/m^2 obtained by Hebrank and Vosburgh (in prep.) for shark skin fibers. An on-fiber unloading curve is not shown in Figure 3 because in general the loading and unloading curves for skin stressed in the direction of the fibers were so close to one another as to be within the limits of resolution of the measuring devices. I do not believe that viscous effects within the fibers make a significant contribution to short-term mechanical behavior.

The stress-strain curves for skin subjected to uniaxial stretching in the hoop and longitudinal directions reveal anisotropy in the mechanical properties of the skin. In both cases the skin deforms by a relatively large amount while the stress remains low, then following this the stress-strain curve becomes steeper as the skin no longer deforms as freely under the applied load. Skin stressed in the longitudinal direction undergoes much greater extensions before it becomes stiff compared to skin stressed in the hoop direction. When the curves become steep, the slope of the curve for skin stressed longitudinally is much less than that for skin stressed in the hoop direction. This difference in slope is equivalent to the difference in elastic moduli, or stiffness, of the skin when stressed in the two directions. The mean terminal elastic modulus (the elastic modulus in the steep part of the stress-strain curve) for skin pulled in the longitudinal direction is 3.5 MN/m^2, which is significantly lower ($F_{(1, 18)} = 5.69$, $0.025 < P < 0.05$) than that of skin pulled in the hoop direction, a value of 14.6 MN/m^2. Values for the mean terminal elastic moduli obtained by stressing skin uniaxially in the on-fiber, hoop and longitudinal directions are compared in Table I.

Breaking stresses and strains for skin pulled in any direction could not be determined. On application of high loads (on the order of 2 MN/m^2 for hoop and longitudinally stressed skin, and 3 MN/m^2 for skin stressed in the fiber direction) the skin always failed at the clips attaching the skin to the testing machine.

In general, skin samples did not return to their original lengths once the load was removed, indicating that some viscous flow within the skin occurred. The hysteresis in the loading and unloading curves further attest to this conclusion. The loss of strain energy is considerably greater for skin stressed in the longitudinal direction.

Stress-strain curves for tests in which one side of the skin was held at a constant stress while the orthogonal side was subjected to an increasing tensile load begin to clarify the nature of the anisotropy noted above. A stress-strain curve for a test performed in which the hoop axis was maintained at a stress of

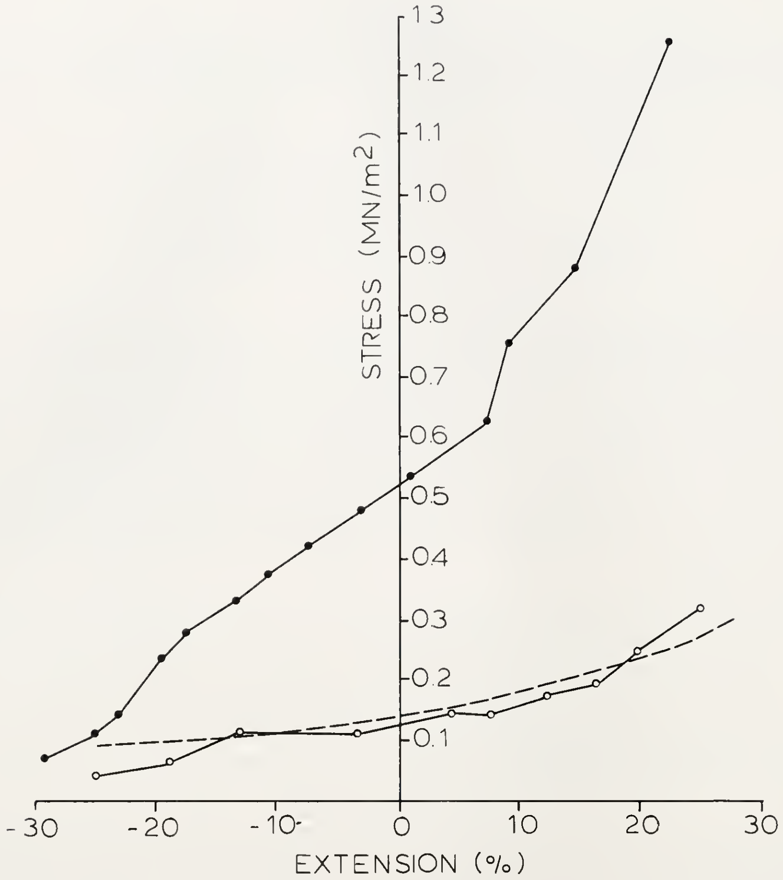

FIGURE 4. Stress-strain curves for tests in which one side of the skin was held at a constant stress while the orthogonal side was subjected to an increasing tensile load. Closed circle, hoop direction pulled while longitudinal direction held at stress of 0.16 MN/m^2; open circle, longitudinal direction pulled while hoop direction held at stress of 0.15 MN/m^2. A theoretical curve for a pure fiber model subjected to the same ratio of forces is shown as a dashed line.

0.15 MN/m^2 is shown in Figure 4. Also in this figure is a graph of a calculated curve, based on the continuous fiber model described previously, subjected to the same ratio of forces applied to the skin. It can be seen from these graphs that the crossed-fibers within the skin behave in a manner similar to the model except at the end points of the curves. In the range of ±10% extension, the elastic modulus of the eel skin was found to average 0.39 MN/m^2, while that of the model shown averages about 0.35 MN/m^2, an insignificant difference. The departure of the experimental curve from the model curve at the end points indicates that the matrix material, the interference between the helical and radial fibers, or some other feature of the skin may become a limiting factor at very high and low fiber angles, or that the density of fibers or their thickness may similarly impose limits to extension.

When the experimental conditions were reversed and the longitudinal axis was maintained at a stress of 0.16 MN/m^2, the stress-strain curve for the hoop direction shown in Figure 4 was obtained. By comparing this curve to that shown for skin stressed in the longitudinal direction and to the theoretical curve, it is readily apparent that skin stressed in the hoop direction does not behave like a pure crossed-fiber system. In the ±10% extension range, the elastic modulus of 13.7 MN/m^2 for hoop extension is two orders of magnitude higher than those obtained for longitudinal extension and the pure fiber model.

Extensions of the skin during swimming. Of eighteen measurements of skin extensions in the circumferential direction, the maximum value obtained was ±11.4% extension. However, in the film analysis it was impossible to tell when the body was exactly straight in the region of the skin markers, so this maximum may be an overestimation of *in vivo* extensions. Most of the measurements fell within the range of ±6–8% extension, so it is felt that this is a reasonable approximation of circumferential skin extensions during swimming. The maximum longitudinal extension measured was ±25.3% ($n = 23$), however longitudinal extensions generally fell between ±10 to 15%. Since the curvature of the eel's body could not be fully incorporated into the measurements, this range of longitudinal extensions may underestimate actual skin extensions by several percent. It should be emphasized that these measurements are meant only to establish an approximate range of *in vivo* extensions of the skin during swimming.

Discussion

The large proportion of collagen fibers within the skin of *Anguilla rostrata* accounts for the skin's strong construction. In consideration of the skin's mechanical properties relevant to locomotor movements, the effects of the radial fibers were assumed to be negligible, and thus only the helical fibers were tested mechanically.

Uniaxial stressing tests illustrate several important properties of crossed-fiber systems. Skin stressed in the direction of the fibers is quite stiff, indicating that the fibers within a skin sample are effectively continuous. The continuity of the fibers is also indicated by the histological evidence; disruptions in the fibers were never observed. Skin stressed in either the hoop or longitudinal directions shows characteristic J-shaped stress-strain curves. Here the skin elongates considerably at low stresses, and this horizontal part of the stress-strain curve corresponds to rearrangement of the two sets of fibers relative to each other, *i.e.*, a change in fiber angle. The steep part of the curve corresponds to more direct loading of the fibers, for at this point the fiber angle has changed so as to nearly align the fibers with the applied load. Now the material deforms less readily under the load, and the elastic modulus increases accordingly.

While skin stressed uniaxially in the hoop and longitudinal directions shows similar qualitative behavior, we have already noted the differences in quantitative behavior, with skin stressed in the hoop direction being stiffer by an order of magnitude than skin stressed in the longitudinal direction.

This anisotropy is more pronounced in the biaxial stressing tests, in which one direction of the skin was held at a constant stress while the stress applied to the orthogonal direction was varied. We have already noted that skin stressed in the longitudinal direction behaves like a pure fiber system, at least within the range of *in vivo* extensions of the skin. Skin stressed in the hoop direction, however, behaves like a composite material of higher elastic modulus than that of the pure fiber model. Clearly something is limiting hoop extension in eel skin.

It is surprising that skin composed of crossed fibers oriented at 45° should be anisotropic in the hoop and longitudinal directions. Here, barring asymmetrical interference from the matrix material surrounding the fibers, the skin should be capable of extending as freely in one direction as in the orthogonal direction. Skin made up of fibers oriented at angles other than 45° would be expected to show anisotropic properties, since the degree of extension is a function of the initial fiber angle (Clark and Cowey, 1958). For example, crossed-fiber helices forming angles higher than 45° with the long axis of the body would resist hoop extensions while permitting longitudinal extensions, and should therefore exhibit a higher elastic modulus in the hoop direction. The fact that eel skin behaves like skin having a fiber angle greater than 45° suggests that other components of the skin make important contributions to its mechanical properties.

If the eel can be thought of as a pressurized cylinder (a reasonable assumption, since muscular contraction most likely creates at least transient pressurization within a myomere), an additional feature within the skin which acts to limit hoop extension would be of benefit to the animal. For a thin-walled cylinder wrapped with helical fibers and pressurized within, the hoop and longitudinal stresses in the walls of the cylinder are balanced when the fiber angle is 55° (Wainwright, Biggs, Currey and Gosline, 1976). This means the pressure within the cylinder may be accommodated without changing the shape of the cylinder; aneurisms will never develop. If the fiber angle is less than 55°, some additional mechanism is required to take up circumferential stresses within the cylinder wall, and it appears that eel skin may have that mechanism.

Mittal and Bannergee (1974), using Verhoeff's staining method, report the presence of elastin fibers intermingled with fine collagen fibers in the dermis of the freshwater teleost *Notopterus notopterus,* however, they give no clue as to the orientation of these fibers. Elastin fibers, if oriented in the direction of the circumference of the eel, might account for the mechanical anisotropy noted, but no elastin fibers were found in this study. It is believed that the protein matrix surrounding the collagen fibers will be found to be responsible for the anisotropy of eel skin, and chemical and mechanical tests are currently being undertaken to determine the nature of the matrix material.

Fujii (1968, p. 102) viewed the primary function of the collagenous layer of fish skin as a means "to strengthen the thin integument of the animal to protect it from splitting under tensile force." From the stressing tests described here we can see that eel skin is strong in tension. Videler (1975, p. 192), in his studies of the cichlid *Tilapia nilotica,* suggested that the skin is too strongly constructed to simply "pliantly conform to the muscle waves." Videler believes that during bending of the fish, the fiber angle does not change, and so forces of equal magnitude must be applied to the skin in the hoop and longitudinal directions. He concludes that tension in the skin must remain constant during a swimming stroke in order for transmission of force from the axial musculature to the tail to be accomplished.

However, both Hebrank (this study) and Wainwright *et al.* (1978) found fiber angles to change during bending. It is these changes in fiber angle that allow the transmission of force through the skin during swimming. In a fish wound with helical fibers, muscle contraction in the anterior region bends the fish, and so the skin on the convex side is extended in the longitudinal direction. This extension, however, produces contraction in the hoop direction as the fiber angle decreases, until these dimensional changes are resisted by the pressurization of the body fluids beneath the skin. Now the skin becomes stiff, and further longitudinal force applied to the skin by the anterior muscles results in tension transmitted to the tail through the skin. In this way the skin of fishes can act as an external tendon, as suggested for sharks by Wainwright *et al.* (1978).

It might appear, however, that an external tendon of this nature would be of less benefit to an eel than it would be to a shark. It should be kept in mind though, that both the eel and the shark swim by undulatory propulsion, and in both cases the amplitude of the waves of bending increases as these waves travel backwards (Gray, 1933), while the mass of the musculature decreases down the length of the body. Thus, in spite of the differences in distribution of the propulsive surfaces, the skin of both fishes may serve a similar locomotor function.

In addition, the eel exhibits another type of locomotor behavior in certain feeding situations. When the eel feeds on something which is too large to swallow, it bites into the food and then rotates about its own long axis several times, with great speed, in order to tear off the mouthful (Tesch, 1977; Moriarty, 1978). This motion can sometimes be seen when an eel is caught on a hook and line, and it has been witnessed in eels in aquaria.

When this "corkscrew" motion occurs, the eel's skin must be stressed in torsion. A cylinder wrapped with inextensible fibers resists torsion most rigidly when the fibers are wound at an angle of 45° with the long axis (the direction of maximum tensile stress during torsion). Thus, when the eel performs its corkscrew motion, the collagen fibers of one helical set (either the right- or left-helical set, depending on the direction of the motion) are being pulled along their long axes. Since the fibers behave as though they are continuous, the tensile force is transmitted along the length of the fish. In this way it is possible that the eel need only generate a twisting moment about the head in order to rotate the whole body. It appears that a fiber angle of 45° is uniquely suited to this corkscrew motion.

Thanks are due to S. A. Wainwright for helpful discussions and advice throughout this study, to J. H. Hebrank for valuable technical assistance, and to C. A. Johnson and F. Hinshaw for supplying most of the animals used in this study. This work was supported in part by a Cocos Foundation Training Grant in Morphology.

SUMMARY

1. The skin of the American eel *Anguilla rostrata* is composed largely of a crossed-helical array of fibers, believed to be collagen, that are oriented at an angle of 45° with the long axis of the fish. Uniaxial and biaxial tension testing of eel skin was used to assess the role of the collagen fibers in the locomotor functions of the skin.

2. The elastic modulus of skin stressed in the circumferential direction of the fish is 14.6 MN/m^2 while that of skin stressed in the longitudinal direction is 3.5 MN/m^2, thus eel skin is an order of magnitude stiffer in the circumferential than in the longitudinal direction. The skin is stiffest in the direction of the fibers, having an elastic modulus of 0.16 GN/m^2 in this direction. Within the range of *in vivo* extensions the behavior of skin stressed in the longitudinal direction is similar to that of a model consisting of continuous crossed-fibers, while that of skin stressed in the circumferential direction is not.

3. The mechanical behavior of the skin indicates that the skin may act as an external tendon, transmitting forces arising in the axial musculature down the length of the fish to the tail.

4. The collagen fibers within the skin may play an important role in torsion resistance when the eel executes its peculiar "corkscrew" motion.

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SOCIAL GROUPINGS AND CIRCADIAN ACTIVITY OF THE KILLIFISH, *FUNDULUS HETEROCLITUS*

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Until relatively recently little was known about the characteristics and controls of circadian rhythms of fishes (Schwassman, 1971a; Thorpe, 1978). Investigations with a number of Arctic species have revealed that the circadian system of fishes is highly labile, adapting to the prevailing environmental conditions (Eriksson, 1978; Müller, 1978a). Studies from more temperate regions have shown that seasonal differences exist in the length of the free running circadian period of the locomotor activity of cyprinid fish (Kavaliers, 1978).

The majority of those studies have dealt with the circadian activity of single fish recorded primarily under constant darkness. However, in the wild many fishes are normally found in groups ranging from aggregates to shoals and schools. What effect group size has on the circadian activity and period of fish and other vertebrates is not well known (Aschoff, 1979). In a brief report Siegmund and Wolff (1973) did suggest that both intensity of illumination and group formation can affect the circadian activity of the European minnow, *Leucaspius delineatus*.

The Atlantic killifish, *Fundulus heteroclitus,* a species of fish that is widely used in physiological and behavioral research, shows evidence of endogenous circadian rhythms. Killifish display a circadian rhythm of color change under constant illumination (Kavaliers and Abbott, 1977). Davis and Bardach (1965) demonstrated a "time coordinated pre-feeding activity rhythm" in killifish that they suggested was determined by an endogenous circadian rhythm. Killifish also occur in shoals and aggregates and thus are an ideal species in which to examine the effects of social grouping on circadian rhythmicity. A shoal is considered to be a facultative social group of fish within which a variety of behavior takes place (Pitcher, 1979). The present study investigates the effects of group size as exemplified by single killifish, aggregates of five fish, and shoals of 25 fish on circadian activity and period length.

Materials and Methods

Killifish were obtained from the Marine Biological Laboratory, Woods Hole, Mass., and placed in 120-liter opaque plastic tanks under a 12-hour light 12-hour dark cycle (LD 12:12) at 20° C (±2° C) and 20‰ salinity. At a random time of day fish were fed a commercial fish food (Purina Trout Chow, St. Louis, Mo.). Activity determinations were carried out in shielded, opaque, polyethylene tanks (125 × 165 × 145 cm) with a gravel substrate and a glass stand-pipe at a water depth of 90 cm. A constant water flow (15 ml/min) at 20° C (± 2° C) and 20‰ was provided by a regulated water supply. Illumination was provided by overhead fluorescent tubes (120 W Sylvania Gro-lite) and incandescent lights (45 W Sylvania), whose intensity could be controlled by neutral density filters (Kodak Wratten) and dimming controls.

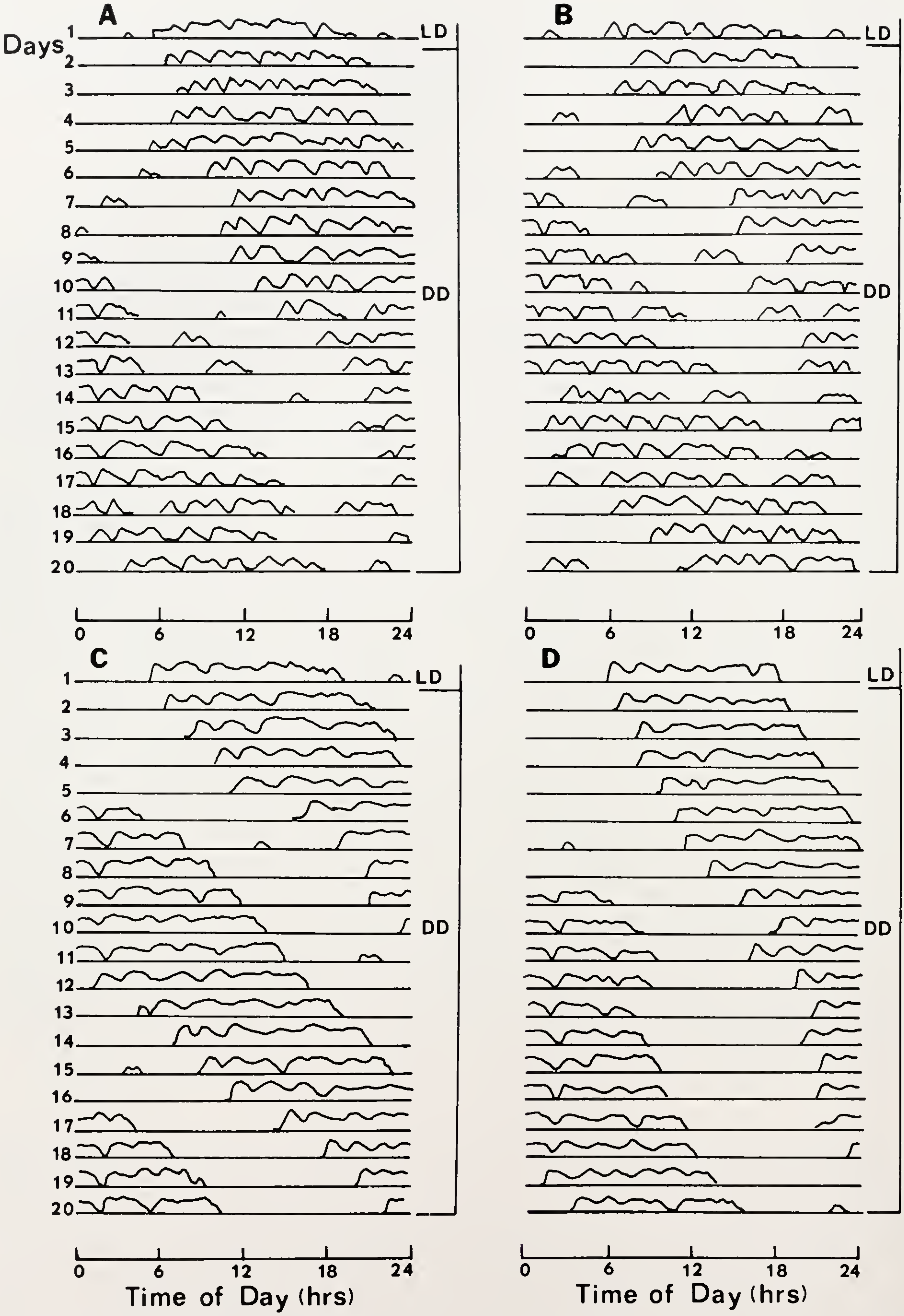

FIGURE 1. Examples of entrained (LD 12:12) and free running (DD, LL) activity totals of killifish. A. group of 5 fish under DD. B. group of 5 fish under LL at 100 lux. C. shoal of 25 fish under DD. D. shoal of 25 fish under LL at 100 lux. Successive days are from top to bottom. Hour 0 is at midnight Eastern Standard Time.

Total activity of individual and groups of fish was recorded by an externally-mounted ultrasonic system described in Kavaliers (1978). For computational purposes activity totals over 15 min were determined. Activity was recorded over 20 to 40 days for all of the individual and groups of fish examined. Visual inspection of the activity records permitted a subjective assessment of the presence or absence of circadian rhythmicity. Power spectral analysis was used to determine whether damped circadian rhythms occurred in the less precise records, as well as to obtain a more exact estimate of period length (Binkley, 1973; Kavaliers, 1978).

Experimental procedures

Ten killifish were individually placed under LD 12: 12 for 10 to 15 days after which constant darkness (DD) was imposed and the length of the circadian period (τ) determined over 15 to 20 days. LD conditions were reimposed and circadian period determinations were repeated under constant illuminations (LL) of 1, 10 and 100 lux. Ten groups of 5 and 25 fish each were placed under LD 12: 12 for 10 to 15 days after which DD and LL (1, 10, 100 lux) were imposed in a similar manner as for the individual fish. Circadian activity and period length were determined for each combination of group size and intensity of illumination.

RESULTS

Circadian activity of individual fish

Under LD conditions individual fish were light-active with maximal activity beginning 5 to 10 min before the onset of light and ending at the dark transition. With the imposition of DD and LL fish displayed a free-running circadian rhythm of activity that lost synchrony with the previous LD condition. Circadian activity of individual fish is described in detail elsewhere (Kavaliers, in prepartion). The relations between and dependencies of the circadian period length on the intensity of LL are summarized in Figure 3. Under DD there was a mean τ of 26.3 ± 0.20 hr . There were no significant differences in period values between different sexes or ages of fish.

Circadian activity of groups and shoals of fish

Five fish. When placed in groups of 5, the fish dispersed through the tank and behaved primarily as isolated individuals, occasionally forming a loose aggregation. Under LD the fish were light-active and under DD and LL they displayed free running circadian rhythms of activity (Figs. 1A, B). There was, however, a significantly more variable period ($P < 0.05$) than that of individual or groups of fish (± 0.50–0.75 hr for the aggregate as compared to ± 0.20–0.40 hr for the individuals and shoals). The increase in variability is shown by the broader and lower amplitude peak in the power spectra (Figs. 2A, B) and the greater variability in the free runs (Figs. 1A, B). Mean or individual group period length was not significantly influenced by LL ($P \leq 0.35$, t-test; 26.0 hr in DD and 25.7 hr in LL, Figs. 1A, B; 2A, B) or the intensity of illumination (Fig. 3).

Twenty-five fish. The group of 25 fish functioned as a facultative cohesive, nonpolarized, interacting social unit or shoal according to the definitions of Breder (1959) and Pitcher (1979). Fish were light active under LD and displayed free running

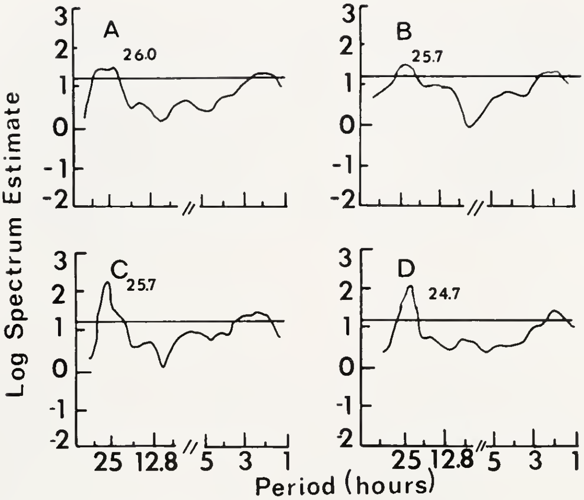

FIGURE 2. Power spectra derived from Fourier analysis of activity records in Figures 1A–D. The horizontal line indicates the upper 95% confidence limit. Only points (periods) above this level are significant. The circadian period associated with the peak is indicated beside.

circadian activity under DD as precise as that of single fish. τ values under DD (25.7 ± 0.20 hr Figs. 3C, 4C) were significantly different from those of single fish ($P < 0.01$, t-test). τ values of the shoals were dependent on the intensity of constant illumination (Fig. 3) (24.7 ± 0.20 hr for 100 lux LL Figs. 1C, D; 2C, D). The intensity or slope of the relationship between τ and LL was significantly different ($P < 0.01$, t-test) from that of the individual fish (Fig. 3). In addition the shoals of fish showed no change or increase in the variability of the period length with increasing intensity of illumination (Fig. 3). The τ values of shoals under LL were significantly less variable (F-test $P < 0.05$) than those of the individual fish.

DISCUSSION

The current study showed that the circadian activity and period length of killifish are affected by group size and social organization. Significant differences exist between the circadian activity and period of individual, aggregates, and shoals of fish, and circadian activity of killifish is affected by the intensity of illumination as per the circadian rule of Aschoff (1960).

According to the circadian rule, light-active animals show a shorter circadian period in LL than in DD, and in LL the period decreases with increasing intensity of illumination. However, in a subsequent reappraisal Aschoff (1979) indicated that various factors can strongly limit the validity of this generalization. In the present study the circadian period of single and shoals of fish complied to the circadian rule, while the group or aggregate of 5 fish failed to show any significant relations. However, the circadian periods of single and shoals of fish also differed significantly from one another. Maintenance of the fish under identical entraining conditions and determinations of τ at the same times of year minimized the possibility of annual variations in τ. In addition, the repeatability of τ values between LD treatments reduced the possibility of spontaneous variations in period length.

Previous determinations of the validity of the circadian rule for fishes have been

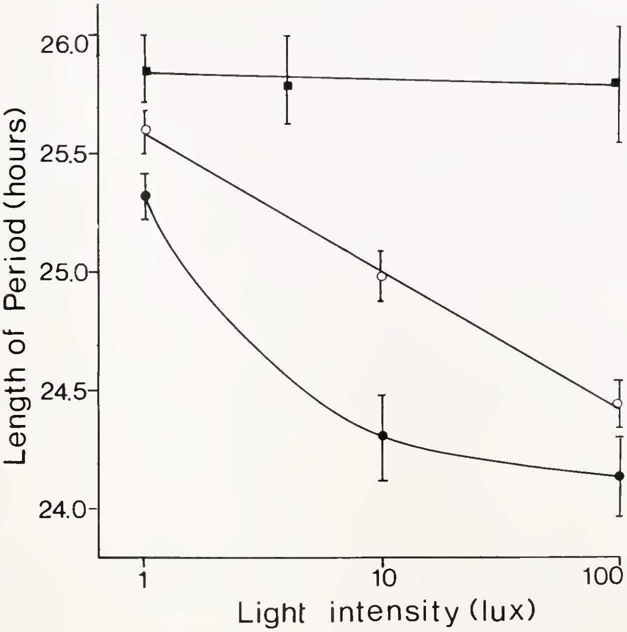

FIGURE 3. Effect of intensity of constant illumination (lux) on the free running circadian period length (τ) of single killifish (closed circles), groups of 5 fish (closed squares) and shoals of 25 fish (open circles). Each point is the mean of 10 indvduals or groups. The vertical line is the 95% confidence limit of the period estimates.

limited to a brief report on the circadian activity of *L. delineatus* (Siegmund and Wolff, 1973) and the frequency of electric discharge of Gymnotid electric fish (Schwassman, 1971b). The validity of the former determinations with *L. delineatus* can be questioned on both procedural and analytic grounds. The τ values were based on values obtained from 2 to 4 days, and were determined immediately after imposition of constant conditions. Sometimes the intial periods are transients (Pittendrigh and Daan, 1976). This may have been the case in the 1973 study of Siegmund and Wolff.

With single fish LL not only significantly increased τ values as compared to DD, but also increased the variability of the period. This is in contrast to the relatively more precise τ values reported for the locomotor activity of birds and mammals under LL. There is often an implicit assumption that the laboratory activity of fish is a single behavior comparable to the circadian parameters of birds and mammals. Fish laboratory activity, however, is a composite of behaviors normally occurring in the wild. Exploratory swimming, feeding, position adjustments and other appetitive and consummatory behaviors of killifish, each showing different reponses to light, cannot be considered as equivalent to the wheel running and perch hopping of mammals and birds, respectively. The latter two are artificial behaviors whose relations to either natural activity or other measures of activity are not established (Aschoff, 1979).

The type and extent of activity displayed by fish is also dependent on the number of fish or group size examined (Welty, 1934; Escobar, Minahan and Shaw, 1936; Symons, 1971). For many fishes and other vertebrates, examination of activity in the absence of conspecifics is an unusual, possibly stressful state.

Physiological and behavioral values determined from single fish need not necessarily bear close relations to values extrapolated from individuals in groups. In the present study the group of 5 fish dispersed through the tank, forming a loose aggregation. Their circadian activity was much more variable than that of single fish and had no significant relation to the intensity of illumination. This reponse of the group of 5 killifish differs from the results reported for *L. delineatus* (Siegmund and Wolff, 1973), though the latter may have formed a shoal. Dispersion of the killifish through the tank may have accentuated individual differences in activity giving a more variable period length. The differences in absolute period length from those of single fish and the lack of compliance to the circadian rule suggests the possibility of additional behavioral or physiological alterations. Aschoff (1979) has indicated that circadian period values are affected by experimental histories, by internal physiological state of the animal and, by external factors other than light, for example group size as shown in the present study.

It is generally assumed that forming a group or increasing the number of individuals in a group leads to a social facilitation of behavior (Clayton, 1978). The feeding behavior of fishes has provided evidence for group stimulation (Allee, 1934) or the so-called "group-effect" (Welty, 1934). This group facilitation arises from exogenous factors, being a function of tank size, density of fish and behavior being investigated (Allee, 1934; Schuett, 1934; Welty, 1934; Symons, 1971). Depending on the density of fish there may be either an increase or decrease in total and individual locomotor activity of groups of goldfish (Escobar, Minahan and Shaw, 1936). Social stimulation and facilitation of the entrainment of circadian activity is suggested for birds (Gwinner, 1966; Menaker and Eskin, 1966). With regard to free running activity a few data suggest that animals kept singly may have a longer period than animals kept in groups (Aschoff, 1979). These responses, however, are derived from experiments that did not involve either direct or visual contact between individuals or social interaction and the formation of groups.

In the present study the group of 25 fish formed a shoal. This was not directly an effect of tank size or density of fish, as in control experiments with larger tanks where density of fish was comparable to that of the individual or the group of 5, shoals were formed. There may be a minimum number of individuals necessary for endogenous social interactions and the formation of a shoal (Breder, 1959, 1976). The shoals of fish were diurnally activity with free running circadian activity under LL that was more precise than that of individual fish and dependent on the intensity of illumination. Müller (1978b) using photoelectric techniques reported that when single and schools of whitefish, *Coregonus laveretus*, were transferred to DD, the single fish became arhythmic while the shoals maintained rhythmicity for up to 10 days. This increase in precision may be considered as a form of social facilitation. However, the significant differences in period values between single and shoals of killifish suggest that the formation of a shoal results in a different behavioral entity with its own circadian characteristics. The circadian activity and period of a single fish cannot be considered as equivalent to, or representative of, an individual in an aggregate, group, or shoal of fish.

Circadian period values recorded after imposition of constant conditions are considered to be or represent history dependent "after-effects" of the previous entrainment conditions (Pittendrigh and Daan, 1976; Aschoff, 1979). Differences in τ values between single and shoals of fish may arise from socially and behaviorally induced differences in LD entrainment, in particular the facilitatory effects of shoal formation and break up at LD transitions.

Killifish as an estaurine species are exposed to temperature, salinity and tidal cycles as well as the daily light-dark cycle. What effects the former factors may have on modifying the circadian period and responses to light are not known. In view of the existence of seasonal changes in the circadian period of other fish (Kavaliers, 1978) the possibility of seasonal variations in the circadian period of single and groups of fish need also to be considered. The effects of social grouping and organization on the circadian rhythms of other fish and vertebrates needs to be further examined.

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SUMMARY

The effects of group size and intensity of constant illumination on the circadian locomotor activity of the Atlantic killifish, *Fundulus heteroclitus,* were examined. Individual fish displayed a free running circadian rhythm of locomotor activity under constant darkness (DD) and constant illumination (LL). The period length (τ) under DD was significantly greater than that under LL (26.3 ± 0.20 hr for DD as compared to 24.6 ± 0.40 hr at 100 lux). Period lengths were significantly dependent on the intensity of constant illumination. Groups of 5 fish displayed more variable circadian activity with no significant differences in τ values from DD and LL. The group of 25 fish formed a shoal and displayed precise circadian periods under DD and LL. The DD period was significantly greater than that under LL (25.7 ± 0.20 hr for DD as compared to 24.7 ± 0.20 hr for 100 lux). τ also decreased with increasing intensity of illumination. Circadian period lengths and relations to LL of shoaling fish were significantly different from those of individual fish. The circadian activity of a single fish cannot be considered as equivalent to, or representative of, an individual in a group or shoal of fish. Social groupings affect both the circadian activity and period length of killifish.

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OXYGEN UPTAKE AND TRANSPORT IN THE PROSOBRANCH MOLLUSC *BUSYCON CANALICULATUM*. I. GAS EXCHANGE AND THE RESPONSE TO HYPOXIA[1]

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The oxygen equilibrium properties of gastropod hemocyanins have been studied *in vitro* since the early work of Redfield, Coolidge and Hurd (1926) and Redfield and Ingalls (1933). These workers showed that oxygen binding of the blood of the prosobranch conch *Busycon canaliculatum* (L.) is cooperative and that the pH dependence of oxygen affinity is reversed relative to that of mammalian hemoglobins. It is now known that oxygen affinity at 22° C decreases from very high to moderately low in the pH range 6.6 to 7.9, above which the large reverse Bohr shift is abruptly replaced by a small normal Bohr shift (Mangum and Lykkeboe, 1979). The physiological meaning of this complex relationship is not clear, however, because the respiratory variables in the blood are largely unknown. The other example of a reverse Bohr shift in a potentially physiological pH range occurs in the xiphosuran arthropods, where the higher oxygen affinity at low blood pH clearly enhances oxygen uptake in hypoxic water (Johansen and Petersen, 1975; Mangum, Booth, deFur, Heckel, Henry, Oglesby and Polites, 1976). Unlike the horseshoe crabs, however, the shelled molluscs have, in direct contact with the tissues, a massive reservoir of $CaCO_3$ that may dissolve and prevent appreciable changes in blood pH (Mangum, Henry and Simpson, 1979).

Quite apart from the response to hypoxia, the respiratory role of gastropod hemocyanin under normoxic conditions cannot be inferred from the information available on other classes in this heterogeneous phylum. Recent studies of the blood oxygen carriers in species representing three of the seven molluscan classes suggest a diversity not found in many animal phyla. In the Amphineura (Redmond, 1962; Petersen and Johansen, 1973) and the nautiloid Cephalopoda (Johansen, Redmond and Bourne, 1978), a relatively small fraction of the oxygen available in the blood is actually delivered to the tissues, and the venous reserve is conspicuously large; this relationship suggests that the blood may be more highly adapted to environmental or behavioral conditions that have not yet been investigated. In the hemoglobin-containing Lamellibranchia, the blood delivers about half of its oxygen load and thus supplies two-thirds of total oxidative metabolism, a common pattern among the various animal phyla (Deaton and Mangum, 1976; Freadman and Mangum, 1976). In contrast, the respiratory role of the blood in either octopod (Johansen, 1965; Johansen and Lenfant, 1966) or decapod (Redfield and Goodkind, 1929) Cephalopoda is truly outstanding in the animal kingdom; very little oxygen remains when the blood leaves the tissues.

We have investigated oxygen uptake and transport in both normoxic and hypoxic water in a member of a fourth molluscan class, the gastropod *Busycon canaliculatum* (L.). The species is found in the lower intertidal and upper subtidal (< 30 m) zones from Cape Cod to Florida, where it reaches maximum abundance on muddy sediments and thus does not overlap with large numbers of its congener *B. carica*.

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MATERIALS AND METHODS

Living material

Specimens of *Busycon canaliculatum* (L.) were obtained from the Chesapeake Bay and various inlets of the Atlantic coast of Virginia. A few observations were made on *Murex fulvescens* Sowerby, from waters near Beaufort, North Carolina, and on *Lunatia heros* (Say), from Woods Hole. Animals were maintained without feeding in natural sea water at 31 to 35‰ salinity and 21 to 24° C. Most of the measurements were made in large aquaria of recirculating sea water, but it was necessary to conduct some of the experiments in the running sea water system of the Wachapreague Laboratory of the Virginia Institute of Marine Science. The animals were always held under the experimental conditions specified for 5 to 10 days prior to a measurement.

Oxygen uptake

Several days before an experiment, the shells were scrubbed and then dipped quickly into melted paraffin wax, followed by iced sea water, to eliminate oxygen uptake by epibiota; as reported previously, this procedure does not cause mortality (Kushins and Mangum, 1971). The animals were placed in a vessel, which was either aerated or flushed with running sea water for several hours until the siphon formed and ventilation resumed. Then the vessel was sealed and the depletion of oxygen monitored with a Yellow Springs Instruments 5420A electrode. Indicator dyes showed that mixing with the self-stirring device was adequate in the smaller (< 500-ml) vessels, but supplementary stirring was provided at the bottom of larger vessels.

The oxygen uptake of various excised tissues was measured with a Gilson differential respirometer, and with a Yellow Springs Instruments Model 53 Cell Respiration apparatus.

Oxygen extraction and ventilation

Animals were suspended and held stationary in the water column in an aquarium of standing water, to allow continuous measurements. P_{O_2} of the excurrent was measured with a Beckman hypodermic microelectrode and Model 160 Physiological Gas Analyzer; P_{O_2} of the incurrent was noted at the same time with the YSI macroelectrode. Ambient P_{O_2} was lowered with N_2 in steps at the rate of about 20 to 30 mm Hg/hr. At each P_{O_2}, several measurements were made for 1 to 5 min each, over a total period of at least 30 min, estimated from the area under a continuous trace.

Water flow was estimated by two methods. First, a thermistor flow probe (LaBarbera and Vogel, 1976) was lowered into the excurrent generated by conchs suspended in a darkened aquarium of standing water, as above. Attempts to record flow into the siphon were unsuccessful because its margins move continuously, touching the probe and inducing the withdrawal response, and also changing both the number and the size of incurrent openings. The records of the excurrent, which is more diffuse but also more constant in shape, were calibrated volumetrically with a Gilson Instruments manometric flowmeter attached to a plastic funnel, made to approximate the dimensions of the exhalant opening. Second, volumetric flow was estimated by attaching the manometer directly to the siphonal canal of the shell. In running sea water, four freely locomoting conchs extended the siphon into the connector tube so fully that a watertight junction was formed. However, they would not ventilate against the resistance of this apparatus unless an open tube was placed in parallel with the manometer: after disconnecting it from the shell, the readings from this Y-shaped apparatus were calibrated with a second manometer.

Blood gases and pH

Many unpaired samples of blood were obtained quickly (< 10 sec) and easily by hypodermic syringe sampling of the large cephalopedal sinus of freely locomoting animals, without apparent harm to the conch. Paired samples of blood were obtained from the afferent and efferent sinuses at the margin of the gill, after inserting the claw of a hammer into the grooves of the shell coils and quickly removing two large pieces before appreciable retraction could occur. Our joint participation was required to complete this operation within 10 to 20 sec of initial contact. Additional, unpaired samples were obtained quickly (< 10 sec) from the ventricles and nephridia of different animals.

Blood gases and pH were measured with a Radiometer BMS1 blood gas analyzer. After air equilibration, oxygen carrying capacity was measured with a Lexington Instruments (Lex-O_2-Con-TL) oxygen concentration analyzer. Because the results seemed anomalous, hemocyanin concentration was estimated from absorbance at 345 nm (Beckman DK-2 spectrophotometer), using the extinction coefficients given by Nickerson and van Holde (1971), and oxygen concentration predicted from Cu content and Cu: O_2 combining ratio (Van Bruggen and Van Holde, 1971).

The blood was equilibrated *in vitro* to various mixtures of N_2, O_2 and CO_2, prepared with Wösthoff pumps. Then its pH was measured with a Radiometer BMS3 pH electrode.

RESULTS

Behavior and mode of gas exchange

In tanks of rapidly running sea water, *B. canaliculatum* often climbs to the surface and extends its siphonal canal into the air. In a more natural posture, however, we were unable to detect any indication of air-breathing. When crawling on sand in an aquarium, conchs responded to a slow lowering of the water level by retracting into the shell. During four days of observing animals in nature at low tide, we invariably found *B. canaliculatum* (30–35 individuals) burrowed partly into the mud so that the siphonal canal was covered. We conclude that, in nature, the mode of gas exchange is exclusively aquatic.

Submerged animals crawl along the bottom, rotating the shell in wide arcs alternately to the left and right. At irregular intervals the pedal muscles contract, elevating the siphonal canal and increasing the volume of the mantle cavity. This motion thoroughly mixes the water in the mantle cavity, possibly enhancing the uptake of oxygen at the mantle and gill. When this response occurred during the measurements of flow and oxygen extraction, the data were discarded.

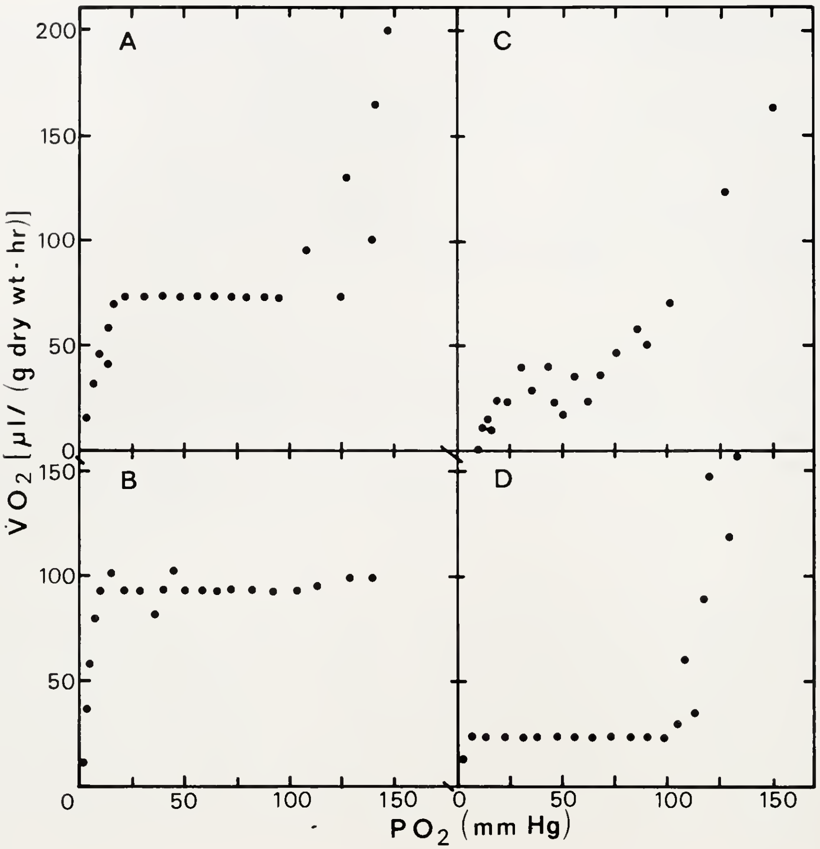

FIGURE 1. The relationship between oxygen uptake ($\dot{V}_{O_2}$) and ambient P_{O_2} in large marine prosobranchs. A. *Busycon canaliculatum* [24-gm(dry wt) animal]. B. Isolated pedal muscle fibers from a specimen of *B. canaliculatum*. C. *Lunatia heros* [5.4-g(dry wt) animal]. D. *Murex fulvescens* [20-g(dry wt) animal] 22 to 23° C, 31 to 32‰ salinity.

Oxygen uptake

The relationship between oxygen uptake and ambient P_{O_2} in *B. canaliculatum*, which is unusually complex, appears to be characteristic of large prosobranch gastropods in warm water (Fig. 1). Essentially there are three distinct components: First, above 120 mm Hg, oxygen uptake changes exponentially with ambient P_{O_2}. The animal usually keeps most of its body inside the shell and does not locomote rapidly, although the siphon is open. The response to mechanical stimulation is conspicuously slow. Secondly, between 40 to 50 and 100 to 120 mm Hg, a range often observed in nature (Deaton and Mangum, 1976), the rate of oxygen uptake is strongly regulated, and behavioral responses to external stimuli are far more rapid. Thirdly, in 17 of 23 animals tested, the rate of oxygen uptake declines below 40 to 50 mm Hg (*e.g.* Fig. 1A).

This complex relationship is found even when the animal is allowed to adjust to the container in running sea water for 24 hr prior to initiating the measurement, and it recurs in as many as three sequential measurements made in tandem. Although no observations of behavior were made, the same pattern was found

TABLE I

Oxygen uptake ($\dot{V}_{O_2}$) of tissue isolated from Busycon canaliculatum. *35‰, 22° C. mean ± s.e.*

Tissue	$\dot{V}_{O_2}$[(µl/(g dry wt·hr)]	N
Pedal muscle	111 ± 4	9
Cardiac muscle	436 ± 17	20
Pedal epidermis	271 ± 14	13
Gill	794 ± 16	17
Nephridium	318 ± 33	17

in two other large gastropods (Fig. 1C, D). In both of the hemocyanin-containing species, *B. canaliculatum* and *M. fulvescens,* oxygen uptake always persists until no oxygen can be detected in the medium, but in *L. heros,* which lacks a blood oxygen carrier, oxygen uptake becomes imperceptible below 10 mm Hg. Despite the production of lactate during anaerobiosis in gastropods (Ellington, Long and Duda, 1977), there is no clear evidence of an increase in oxygen uptake following brief exposure to hypoxia during the second or third of the sequential measurements.

At 21 to 23° C, 31 to 34‰ salinity and ambient $P_{O_2} = 100$ to 120 mm Hg, the mean value for oxygen uptake in 12 animals of intermediate body size (22–27 g dry wt) is 82 (± 4 s.e.) µl/(g dry wt·hr). The maximum rate at $P_{O_2} > 140$ mm Hg, which is far more variable, is 200 (± 23 s.e.) µl/(g dry wt·hr). The weight-specific rate of oxygen uptake by most of the isolated tissues in air saturated water exceeds that of the animal as a whole (Table I, Fig. 1) by a large margin. This relationship could be explained alternatively as follows. Either, $\dot{V}_{O_2}$ (whole animal) is an average of the values in Table I, weighted according to the relative mass of each tissue. This explanation requires the condition that the pedal muscle comprises more than 2/3 of total body weight, which is untrue. Or, $\dot{V}_{O_2}$ (whole animal) is an average of the values for the various tissues, weighted according to their biomass and also according to their ambient (*viz.* blood) P_{O_2}. This explanation, which is far more consistent with tissue weight distribution, requires the condition that the oxidative demand of most of the tissues in an animal is not fully satisfied by the supply. This hypothesis is strongly supported by measurements of oxygen uptake in pedal muscle fibers (Fig. 1); in none of the 4 experiments was the rate maintained below 10 mm Hg.

Ventilation

The water current enters the mantle cavity through the siphon, a tube formed transiently by the mantle. The current enters the mantle cavity and then passes obliquely over the head and gills. After flowing over the excretory openings, the water exits the shell from the side of the mantle cavity opposite to the siphon, as a diffuse current. Extensive mixing in the excurrent is often observed when particles are present in the water.

In addition to ventilating the two sites of oxygen uptake into the blood, the current flushes poorly vascularized epithelium. Oxygen is also taken up from water that is not actively moved by the animal, at the leading and trailing surfaces of the foot, and the rate of oxygen uptake by these epithelia is relatively high (Table I). Thus, not all of oxidative metabolism is fueled by the diffusion of oxygen from the actively generated water current into the blood.

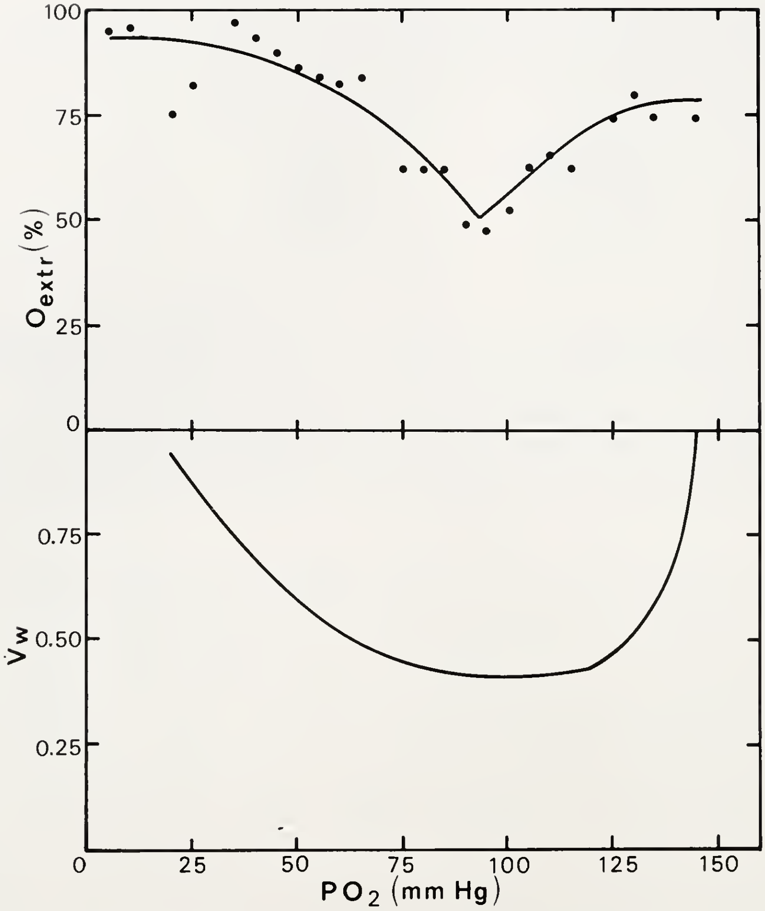

FIGURE 2. The relationship between oxygen extraction (O_{extr}, top), ventilation ($\dot{V}_w$, bottom) and ambient P_{O_2} in *Busycon canaliculatum* (22–24° C, 32–34‰ salinity).

The flow rate varies considerably over short periods, which might lead to appreciable errors in estimating ventilation from point observations with a manometer. On the other hand the calibration of the records made from the diffuse excurrent requires precise simulation of the cross-sectional area of the current, which is difficult. In addition, the posture of the animals was not the same during the two sets of measurements. Nonetheless, the agreement between the data obtained with the two methods is quite good, and we suggest that they are reasonably accurate. The most stable value obtained with the manometer is 15 ml/(g dry wt·hr), and the value estimated from the integral of the continuous record is 14.3 (± 1.1 s.e.) ml/(g dry wt·hr) (N = 9). The rate predicted from the data for total $\dot{V}_{O_2}$ and per cent O_2 extraction is 18.3 ml/(g dry wt·hr). The discrepancy may be due to an assumption involved in making this calculation, *viz.* that all of oxygen uptake is fueled by active ventilation, which is incorrect. The difference of about 15 to 20% may represent the passively ventilated component of oxygen uptake.

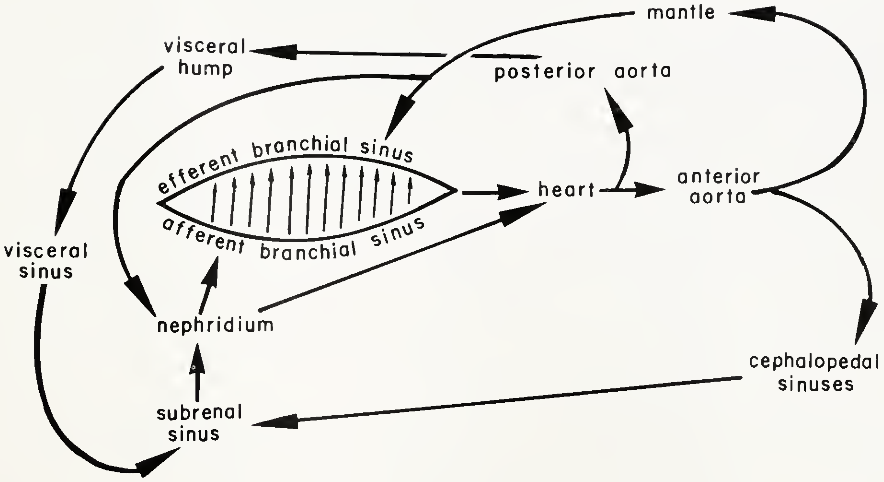

FIGURE 3. The path of blood flow in *Busycon canaliculatum*.

Oxygen extraction

The complexity of the relationship between per cent oxygen extraction and ambient P_{O_2} parallels that between $\dot{V}_{O_2}$ and P_{O_2} (Fig. 2). The two relationships are, of course, interdependent. In the range 100 to 159 mm Hg, both O_2 uptake and O_2 extraction change exponentially. At 100 mm Hg, O_2 extraction reaches the level expected in an animal that generates a respiratory and not a trophic water current (Mangum, 1977). At 25 to 100 mm Hg, oxygen extraction steadily increases, thus permitting the regulation of oxygen uptake in spite of the decreasing oxygen supply in the medium.

No measurements of water flow were obtained at low P_{O_2}, due to behavioral responses that interfered with each of the two methods. The relationship between $\dot{V}_w$ and ambient P_{O_2} can be predicted, however, from the data for $\dot{V}_{O_2}$ and per cent O_2 extraction, assuming no effect of P_{O_2} on the partitioning of oxygen uptake between actively and passively ventilated gas exchange sites. This relationship is shown in Figure 2 in arbitrary units, because the absolute values would be somewhat high (see above).

Circulation of the blood

The pattern of blood flow is diagrammed in Figure 3, which is based on the accounts of the anatomy of the system by Dakin (1912) and Pierce (1950), and a few personal observations. Blood emerges from the heart into an aorta which immediately divides into two branches. The smaller of the two, the posterior or ventral aorta, supplies the deep visceral tissue, intestine and gonad, that fills the innermost coils of the shell. Blood returning from these tissues collects into a perivisceral sinus and then passes to the subrenal sinus and the nephridium. Blood leaving the nephridium may pass into the gill or directly into the auricle as in other molluscs. The much larger anterior or cephalopedal aorta gives off a pallial branch and then proceeds along the esophagus, where its additional branches supply the feeding and digestive organs, and into the foot and head regions, where it enlarges to form a secondary pump. The blood leaving the digestive, sensory

TABLE II

Respiratory properties of the blood of Busycon canaliculatum. *22 to 24° C, 32 to 34‰ salinity, ambient P_{O_2} 100 to 120 mm Hg, ambient P_{CO_2} 1.0 mm Hg. mean ± s.e. (N).*

Blood pH	
postbranchial	8.04 ± 0.09 (4)
pedal	7.86 ± 0.01 (23)
prebranchial	7.85 ± 0.01 (4)
Blood P_{O_2} (mm Hg)	
postbranchial	27 ± 7 (9)
cardiac	21 (2)
pedal	5.5 ± 1.3 (10)
renal	5.3 (2)
prebranchial	2.3 ± 0.4 (11)
Blood P_{CO_2} (mm Hg)	
postbranchial	1.1 (2)
prebranchial	1.8 (2)
Blood ammonia (mM/liter)	
postbranchial	0.03 ± 0.01 (6)
pedal	0.08 ± 0.02 (18)
prebranchial	0.09 ± 0.01 (6)
Blood O_2 carrying capacity	3.68 ± 0.29 (12)

and pedal structures collects in the large cephalopedal sinuses, and passes from there into the subrenal sinus, through the nephridium and then either to the gill or heart. Thus the blood travelling this route, which appears to be diluted with sea water in the foot (Mangum, 1979), may return to the tissues without being oxygenated, but not without being filtered. Although Pierce (1950) makes no mention of the pallial branch of the anterior aorta, a third major route of circulation, it is clearly visible in *Busycon* as a large vessel that extends into superficial epithelium of the mantle. If its position in the system is the same as in the related neptuneid conch *Buccinum* (Dakin, 1912), it branches off before the anterior aorta reaches the esophagus and extends throughout the dorsal part of the mantle and the siphon. Blood returning from the mantle, a site of oxygen uptake, enters either the heart or the efferent branchial sinus, and thus mixes with the blood leaving the gill, a second site of oxygen uptake. According to Dakin (1912), some of the blood travelling the pallial route is also filtered by the nephridium before returning to the heart.

Blood pH and buffering

As the blood flows through the circulatory system, the pH change is exceptionally large for an aquatic animal (Table II). The largest change appears to occur between the gill and the pedal sinus; there is little further net increase in acidity as the blood is filtered and returned to the gill.

The reverse Bohr shift contributes to the large pH change (Fig. 4); unlike bloods containing carriers with a normal Bohr shift, deoxygenation results in an increment in acidity. However, the effect of oxygenation state on pH is small. Similarly, blood ammonia levels are always low, as is the rate of ammonia excretion (Polites and Mangum, 1979). In contrast, the change in total CO_2 as the blood travels through the system (> 0.04 mM) is more than sufficient to explain the

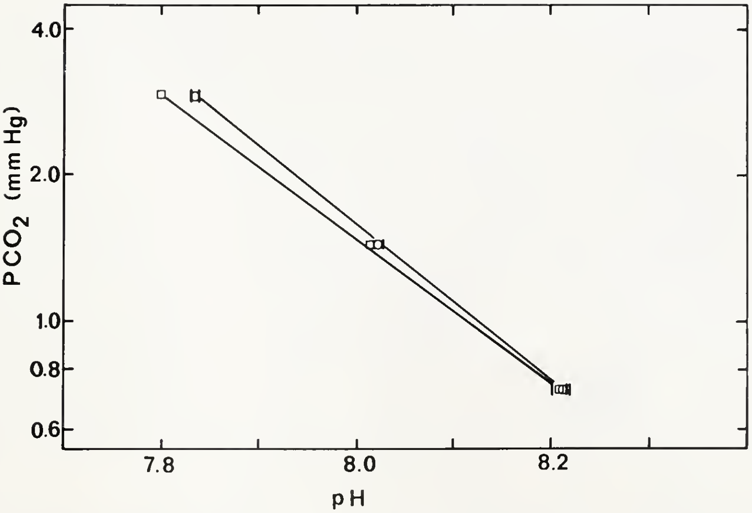

FIGURE 4. Buffer properties of oxygenated (open circles) and deoxygenated (open squares) blood taken from *Busycon canaliculatum* (22° C, 31‰ salinity).

observed change in pH. Whether the relatively large CO_2 change is due to a slow rate of blood flow or, equally likely, to the special CO_2 requirements of shell secretion, is not clear, but we suggest that the primary agent of the pH change is the exchange of a component of the CO_2 system.

When animals are held in hypoxic water ($P_{O_2} < 20$ mm Hg) for 6 hr, pedal blood pH (7.82 ± 0.03 s.e.) and P_{CO_2} [1.5 (± 0.2 s.e.) mm Hg] change very little ($N = 6$). Moreover, there is no increase in free Ca^{+2} content of the blood (Mangum and Lykkeboe, 1979), suggesting that the shell is not an important source of buffer under these conditions. When exposed to hypoxia for longer periods (12–24 hr), pedal blood pH dropped to 7.4 to 7.6. However, none of six animals in each of two experiments survived this experience, and no further measurements were attempted.

Oxygen carrying capacity

Numerous samples of blood from the pedal sinus of freely locomoting animals yielded values for oxygen carrying capacity that were unexpectedly low [1.19 (± 0.06 s.e.) ml/100 ml, $N = 58$]. They were, however, consistent with predictions based on hemocyanin concentration [1.39 (± 0.07 s.e.) ml/100 ml, $N = 11$]. Surprisingly, the values for samples taken from the nephridium of the same animals were 2 to 4 times higher. This apparent anomaly occasioned a more intensive study of the changes in hemocyanin levels in different parts of the circulatory system and in the water extruded from the shell as the animal withdraws, which was undertaken in the closely related species *Busycon carcia* because of its greater abundance (Mangum, 1979). Although fewer data are available for *B. canaliculatum* (Table II), they are entirely consistent with the findings for *B. carcia:* when the animal inflates its foot, pedal blood appears to be diluted, presumably with sea water, and the blood is reconcentrated before returning to the sites of oxygen uptake. The figures for oxygen carrying capacity given in Table II represent branchial, cardiac and nephridial blood.

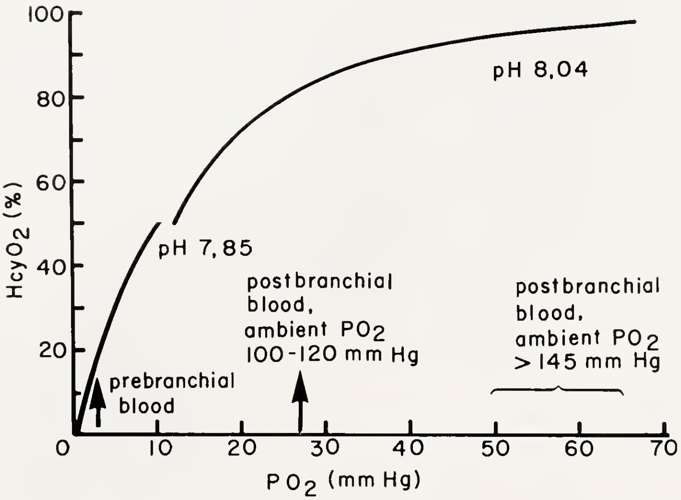

FIGURE 5. Oxygen equilibrium of the blood taken from *Busycon canaliculatum* at postbranchial and prebranchial blood *p*H (22° C, 31‰ salinity). From data reported by Mangum and Lykkeboe (1979). Brackets show range of blood P_{O_2} when ambient water $P_{O_2} = 145$ to 159 mm Hg.

Blood P_{O_2} and oxygenation

When animals are held at an ambient P_{O_2} of 100 mm Hg, where the rate of oxygen uptake is stable, blood P_{O_2} at the efferent margin of the gill usually falls within the range 20 to 30 mm Hg. At ambient $P_{O_2} = 145$ to 159 mm Hg, the values are far more variable, although they fall in the range 50 to 65 mm Hg when the siphon is clearly protruded. As expected from the mass of tissue supplied with oxygen at a particular site in the circulatory system, the largest decrease in blood P_{O_2} occurs in the pedal sinus (Table II), and the further change is relatively small. Prebranchial blood P_{O_2} varies very little, regardless of the oxygen level in the medium.

In air-saturated water, the hemocyanin in the blood is essentially oxygenated in full at the gill (Fig. 5), and at 100 to 120 mm Hg the per cent oxygenation is almost as great. Although most of the O_2 load is delivered before the blood leaves the pedal sinus for the nephridium, the change in oxygenation accompanying the small drop in P_{O_2} between the foot and the gill results in the delivery of about 0.3 ml O_2/100 ml blood, which is a substantial supply for the nephridium, a relatively small mass of tissue. The venous reserve of O_2, or the amount remaining in the blood as it returns from the tissues, is exceptionally small. In view of the low estimate of blood flow (Table III), which implies a long equilibration time at the tissues, and also the locomotor capability of the animal, which was essentially maximal under the conditions studied, this finding is not surprising.

DISCUSSION

In comparison with the other molluscs studied, the rate of total oxidative metabolism is higher than expected in *Busycon canaliculatum*, especially considering its low level of motor activity in relation to many other molluscs (Table III). Comparing the regulated rates, which we suggest are more meaningful, *B. canaliculatum* has a higher capacity for oxidative metabolism (2–3 times) than the

TABLE III

Respiratory variables in six species of molluscs. Data for Octopus, Nautilus *and* Cryptochiton *converted by assuming that dry wt = 20% wet wt. Ambient P_{O_2} (PiO_2) > 140 mm Hg unless specified otherwise.*

	Busycon canaliculatum	*Cryptochiton stelleri*	*Noetia ponderosa*	*Modiolus demissus*	*Nautilus pompilius*	*Octopus dofleini*
Body wt., dry (g)	24	*ca.* 80	5	1.35	*ca.* 116	*ca.* 2020
Experimental temperature (°C)	21–23	10	21–23	21–23	16–20	8–11
$\dot{V}_w$ [ml/(kg dry wt·min)]	238–250	*ca.* 380	267	2233	*ca.* 1480	*ca.* 1360
O_2 extraction from water (%)	50*	48	60	8–9	7	80
$\dot{V}_{O_2}$ [ml/(kg·min)]	1.17*	*ca.* 0.33	5.27	7.88	*ca.* 2.25	*ca.* 1.97
Blood O_2 carrier	Hcy	Hcy	Hb	none	Hcy	Hcy
% oxygenation						
postbranchial	84*	93	99		99	94
prebranchial	16*	74	52		63	21
Blood O_2 delivered to tissues (ml/100 ml)	2.25*	0.36	1.82	0.06	1.1	2.9
O_2 extraction from blood (%)	84	48	47	14	*ca.* 40	80
Cardiac output [(ml/(kg·min)						
Fick principle	52*	92	125	1313	205	68
heart rate × volume	26*			1241		
Source	present data, deFur and Mangum (1979); Mangum and Lykkeboe (1979)	Redmond (1962) Petersen and Johansen (1973)	Deaton and Mangum (1976), Freadman and Mangum (1976)	Booth and Mangum (1978)	Redmond, Bourne and Johansen (1978), Johansen, Redmond and Bourne (1978)	Johansen (1965), Johansen and Lenfant (1966)

* PiO_2 = 100 to 120 mm Hg.

other large prosobranchs examined, including the hemocyanin-containing *Murex fulvescens* (Fig. 1). Members of the genus *Murex* have an appreciably lower rate (38%) of oxygen extraction as well (Hazelhoff, 1939).

The high capacity for oxidative metabolism does not result from a high rate of oxygen convection on either side of the respiratory epithelium (Table III). Oxygen extraction, both from ambient water and from blood, is quite great, which emphasizes the importance of the relatively long equilibration periods accompanying low rates of fluid flow, in spite of the smaller P_{O_2} gradient driving oxygen into the tissues. The oxygen-carrying capacity of *Busycon* blood is also relatively high, although none of the bloods with hemocyanin reach the capacity of the hemoglobin-containing blood of the ark clam *Noetia*. We suggest that oxygen uptake in *B. canaliculatum* is also enhanced by the more or less serial arrangement of the two sites of oxygen uptake, the mantle and the gill, which greatly extends the distance over which oxygen can be extracted from the water current. Positioned first in the series where they extract the most highly oxygenated water are the smaller pallial vessels and then the gill, which essentially act as preamplifiers of blood oxygen. Next in the series are the larger pallial vessels, which discharge into the efferent branchial sinus. The importance of this arrangement can be inferred from the data for normoxic water. At an incurrent water P_{O_2} of about 130 to 159 mm Hg, oxygen extraction is 75%; the P_{O_2} of the excurrent is 33 to 40 mm Hg. And yet postbranchial blood P_{O_2} reaches levels of 50 to 65 mm Hg. The inverse relationship between water and blood P_{O_2} can be possible only if the blood at the first site of oxygen uptake is considerably more oxygenated than the blood at the second site in the series, and/or if a very large fraction of additional oxygen is removed after the current passes the two sites of oxygen entry into the blood.

The mantle is exquisitely sensitive to mechanical stimulation, and we were unable to estimate its gas exchange surface in an animal assuming its ventilation posture. However, we must emphasize that the figure of 8 cm² gill surface area/gm wet wt (Ghiretti, 1966), only 10 to 15% lower than in other molluscs with a non-trophic gill, vastly underestimates the total surface available for gas exchange in neptuneid conchs.

The strong regulation of oxygen uptake in the ambient P_{O_2} range 40 to 50 to 100 to 120 mm Hg is due in part to the equally strong regulation of heart rate (de Fur and Mangum, 1979). Unless the partitioning of oxygen uptake between actively and passively ventilated sites changes with ambient P_{O_2}, however, regulation is also due to the increased ventilation predicted by the data for oxygen extraction and oxygen uptake. Such a pronounced response by a ciliary pump is somewhat unexpected, both on *a priori* grounds and on the basis of direct measurements of water flow; in lamellibranchs water flow increases only slightly at intermediate oxygen levels and then sharply falls in hypoxic water (Booth and Mangum, 1978). We cannot dismiss the possibility that our assumption of an unchanged partitioning of oxygen uptake is incorrect. Alternatively, the flow rate may be regulated by a variable under direct control by the central nervous system, such as the geometry of entrance conditions, or the shape of the siphon.

In Table III cardiac output was calculated according to the Fick principle, using the data for total oxygen uptake and the change in oxygen concentration as the blood passes the tissues. The validity of this calculation requires a condition which is clearly untrue, *viz.* that all of the oxygen consumed is first taken up by the blood. Not surprisingly, in view of the large surface area of unvascularized tissue exposed directly to the ambient medium, the result is considerably higher than the figure obtained by an alternative method. DeFur and Mangum (1979) calculated cardiac output from measurements of heartrate times stroke volume, which was deliberately estimated on the conservative side. Although the alternative figure may be somewhat low, it is unlikely to be in error by as much as 50%, and the discrepancy is far more likely to be real, due to the uptake of oxygen by unvascularized epithelium, the rate of which is relatively high (Table I).

The adaptive significance of the reverse Bohr shift in *B. canaliculatum* differs from that in *Limulus* (Johansen and Petersen, 1975; Mangum *et al.*, 1976). Unless the effect of hypoxia on branchial blood pH is fundamentally different from the response of foot blood pH, which does not change significantly until the animal is moribund, then the reverse Bohr shift does not facilitate oxygen uptake in hypoxic water, as it does in *Limulus*. Indeed, oxygen uptake declines sharply in hypoxic water (Fig. 1A). In normoxic water, the change in blood pH at the gill induces a normal Bohr shift which is too small to be physiologically significant. The important point is that the reverse Bohr shift is not the physiological problem often supposed in the past, because blood pH falls below 7.9, the critical value at which the reverse Bohr shift abruptly appears, only slightly and only after the blood has delivered three-quarters of its oxygen load. As suggested earlier (Mangum and Lykkeboe, 1979), the reverse Bohr shift may even be an advantage at this point, because it opposes further depletion of the already small oxygen load in the blood, which must still supply the nephridium before returning to an oxygenation site.

There may be another effect of the decrease of blood pH below 7.9 which is more important than the respiratory effect. The abrupt change from normal to reverse Bohr shift is apparently due to the aggregation of 4.5 × 10^6 dalton particles to 9×10^6 dalton particles (DePhillips, Nickerson and Van Holde, 1970). The aggregation state of *B. canaliculatum* hemocyanin is also influenced by deoxygenation, which increases the number of 9×10^6 dalton particles formed. While the rate of aggregation of half particles at pH 8.3 and in artificial media is very slow (> 6.5 hr), the kinetics of disaggregation and of the pH effect are not known. It is possible that the aggregation state changes *in vivo* as the blood flows through the system, which would also result in appreciable changes in the activity of the inorganic ions in the blood (Mangum, unpublished data). Fluid balance between the tissues and either the blood or ambient sea water could be altered by one or both of two mechanisms: the doubling of the colloid osmotic pressure of the hemocyanin particles *per se* and the colligative behavior of the inorganic osmolytes bound or released. These two properties of the blood are presently under investigation by the authors.

The data in Figure 4 were collected by G. Lykkeboe, in collaboration with the authors.

SUMMARY

1. The blood of the conch *Busycon canaliculatum* is highly oxygenated at the two sites of gas exchange with the ambient medium, the gill and the mantle. An exceptionally small venous reserve is maintained in prebranchial blood, suggesting an important respiratory role of the oxygen transport system.

2. In normoxic water the physiological pH range is 7.85 to 8.04, where the reverse Bohr shift of *Busycon* hemocyanin is essentially absent. During non-lethal exposure to hypoxia, blood pH does not decrease substantially and thus the reverse Bohr shift cannot be interpreted as an adaptation to low oxygen conditions.

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ECDYSONE METABOLISM AND THE INTERRUPTION OF PROECDYSIS IN THE LAND CRAB, *GECARCINUS LATERALIS*[1]

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In the brachyuran crabs, there is a complex interaction between limb regeneration and the intermolt cycle. The majority of limb regeneration occurs during the premolt period of the intermolt cycle (proecdysis; stage D) during which the animal undergoes preparation for ecdysis. However, regeneration can, in turn, influence the progress of proecdysis: autotomy of limbs or partially regenerated limbs before a critical stage in proecdysis (stage D_1) results in a temporary suspension of proecdysis while a new round of regeneration occurs at the sites of the missing limbs. During this suspension in stage D_0, not only is there a cessation of the growth of partial regenerates remaining on the crab (Holland and Skinner, 1976), but gastrolith deposition and the cytological activation of the epidermis are also delayed. This suspension of proecdysis is associated with a rapid decline of serum ecdysteroid titers from ~ 70 to ~ 15 ng/ml within a day of the autotomy; ecdysteroid titers remain low for two weeks while the recently autotomized limbs undergo basal regeneration. Titers then re-initiate their normal pattern of proecdysial increases; events which had been temporarily suspended resume development in association with the increased hormone titer so that the animal undergoes a normal, but delayed, ecdysis and releases a full compliment of regenerated limbs (McCarthy and Skinner, 1977b).

This interaction between regeneration and the molt cycle suggests that changes in the titer of 20-hydroxyecdysone, the biologically active ecdysteroid, regulate the progress of proecdysial events; and that the accepted paradigm of molt regulation (Passano, 1960), is incomplete, since these same interactions occur even in animals deprived of eyestalks, the source of the molt-inhibitory hormone (McCarthy and Skinner, 1977b).

In this paper, the metabolic fate of ecdysone [α-ecdysone, the secretory product of the Y-organ (Chang and O'Connor, 1977)] is examined in animals in late stage D_0 either with or without the simultaneous autotomy of four partially regenerated limbs. The purposes of this study are: to support and extend our earlier studies on ecdysteroid metabolism in crabs in the intermolt (stage C_4; McCarthy and Skinner, 1979) and mid-to-late premolt (stages D_1 to D_2 and stage D_3; McCarthy, in preparation) stages of the intermolt cycle; to examine the biochemical mechanisms by which the rapid decline in ecdysteroid titers is achieved following limb autotomy in stage D_0; and to provide additional information on the nature of the regulatory mechanisms responsible for the interruption and re-initiation of proecdysis.

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MATERIAL AND METHODS

Animals

Specimens of the land crab, *Gecarcinus lateralis,* ranging in weight from 30 to 60 g were obtained from the Bermuda Biological Station and were maintained in the dark and fed weekly. The animals were induced to enter proecdysis precociously by causing the animals to autotomize 8 walkings legs (Skinner and Graham, 1970, 1972). Premolt animals in late stage D_0 were used. They were determined to be in late stage D_0 by the following criteria: limb regenerates were at an R-value between 14 and 17 (Bliss, 1956; Skinner, 1962); serum ecdysteroid titers [estimated by radioimmunoassay (RIA); Horn, Wilkie, Sage and O'Connor, 1976] were between 60 and 90 ng/ml (McCarthy and Skinner, 1977a, b); and the epidermis was still firmly attached to the cuticle of the branchiostegite region. Crabs met these criteria approximately 30 days after molting was induced by multiple limb autotomy.

To examine the mechanisms by which endogenous ecdysteroid titers are rapidly decreased when limb regenerates are re-autotomized in stage D_0, another set of metabolic studies was conducted on crabs meeting the above criteria for stage D_0; however, in these animals, four of the partially regenerated limbs were re-autotomized immediately before the [^{3}H]ecdysone was injected (at $t = 0$ hr in Figs. 1B, 3, 4B). This group of animals are designated as "LBA crabs" (limb bud autotomized). Crabs in stage D_0 which did not have limbs re-autotomized are sometimes referred to as "normal crabs in stage D_0" to distinguish them from the LBA crabs.

Injection and extraction of ecdysones

A small mass of high specific activity α-[23,24 ^{3}H]ecdysone ($\sim$ 55 Ci/millimole) was diluted in perfusion fluid (Skinner, Marsh and Cook, 1965) and a total volume of 2–3 μl/g live wt. (0.3–0.5 ng ecdysone/g live wt) was injected into the hemolymph sinus at the base of a chela. The [^{3}H]ecdysone, which was a gift of Dr. D. S. King (Zoecon Corp., Palo Alto, CA), was chromatographically purified prior to use (see below). At 1, 4, 9 or 24 hr after injection crabs were chilled on ice and dissected. Samples of hemolymph (from the chela sinus) and epidermis (from the branchiostegite region) were removed as were midgut gland (including the midgut) and the hindgut. These tissues were rinsed in saline and they, as well as the remainder of the crab, referred to as "carcass," were frozen in liquid nitrogen and held at $-60°$ C.

For each time point tissues from 3 to 4 crabs were pooled and extracted 3 times in hot methanol. The pooled extracts were partially purified as previously described (McCarthy and Skinner, 1979). This crude purification procedure permitted recovery of 65 to 75% of the total radioactivity.

Chromatographic separation of ecdysteroids

Ecdysone metabolites in the partially purified extract were separated by high pressure reverse phase liquid chromatography (HPLC) on a 3.9×60-cm μBondapak C-18 column (Waters Assoc.) eluted with a linear gradient of 20 to 100% methanol in water over 165 min at a flow rate of 0.5 ml/min. A Model 6000 solvent delivery system with a Model U6K universal injector and a Model 660 solvent programmer (Waters Assoc.) were used for the analyses. The relative

Table I

Ecdysone and its metabolites in normal crabs in stage D_0. Dashes indicate that the compound was not detected; "NR" is the very polar compound which was not retained by the column even at 20% methanol.

Tissue	Hr after injection	Component (% radioactivity)						
		NR	Polar conjugate of			Free ecdysteroids		
			20,26-OH	β	α	20,26-OH	β	α
Serum	1	—	—	—	—	—	58	42
	4	—	—	—	2	13	81	4
	9	—	—	—	—	16	82	2
	24	—	—	—	—	14	85	1
Epidermis	1	—	—	1	—	1	50	48
	4	—	—	5	—	5	83	7
	9	—	1	10	—	17	69	3
	24	—	6	—	—	13	80	1
Carcass	1	—	3	—	9	11	29	48
	4	—	—	—	5	6	65	24
	9	—	—	—	—	14	72	16
	24	—	—	—	—	15	83	2
Midgut gland	1	—	—	7	72	—	4	17
	4	—	—	20	43	—	15	22
	9	—	—	30	39	3	12	16
	24	—	4	15	15	13	18	15
Hindgut	1	—	—	—	—	—	42	58
	4	—	—	10	10	5	74	11
	9	—	—	2	2	16	77	5
	24	15	—	—	—	13	72	—
Total crab	1	—	1	11	11	6	38	44
	4	—	—	9	9	7	64	18
	9	—	—	6	6	13	64	12
	24	—	1	3	3	15	70	4

contributions of the various metabolites were determined by liquid scintillation counting of 1-ml fractions of the eluent. Details of these procedures have been described (McCarthy and Skinner, 1979).

Rate coefficients

The rates of conversion, turnover and elimination of ecdysone metabolites are expressed as rate coefficients, k, estimated from the slope of the semilog disappearance curve (*e.g.*, Fig. 1). In cases where the semilog plot was clearly non-linear the rate constants for the fastest and slowest components were estimated by graphical analysis (Atkins, 1969). The data in Figures 2 and 3 are not presented as semilog plots; data were replotted for graphical analysis. The rate coefficients are expressed in inverse time and, in most cases observed in this study, varied from 0.002/hr ($t_{1/2} = 350$ hr) to 0.10/hr ($t_{1/2} = 5.6$ hr).

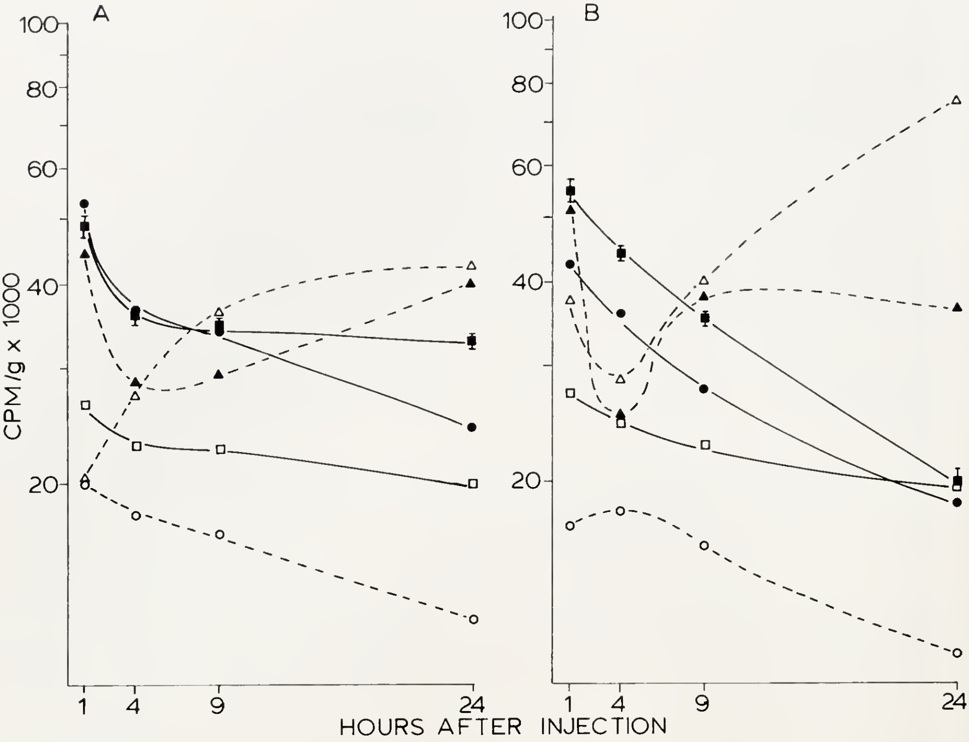

FIGURE 1. Concentration of total radiolabeled ecdysone and its metabolites following injection of [^{3}H]ecdysone in (A) normal crabs in stage D_0 and (B) LBA crabs in stage D_0 (with partially regenerated limbs autotomized at t = 0 hr). The pattern of change is indicated for serum (closed boxes), epidermis (closed circles), hindgut (closed triangles), total crab (open boxes), midgut gland (open triangles), and carcass (open circles). As an estimate of the variability within experimental groups, the radioactivity in the serum of individual crabs was determined and results are plotted as the mean ± 1 SEM.

RESULTS

Metabolites of ecdysone

[^{3}H]Ecdysone was very rapidly converted by tissues of crabs in stage D_0 to a total of five metabolites, two of which co-chromatographed with authentic 20-hydroxyecdysone and 20,26-dihydroxyecdysone (see Fig. 1 in McCarthy and Skinner, 1979). Three radiolabeled polar components were also observed. These components have been identified in intermolt crabs as polar conjugates, probably glycosides, of the three free ecdysteroids (McCarthy and Skinner, 1979). In the hindgut, a sixth radiolabeled metabolite was detected which was very polar and eluted from the reverse phase column in the void volume. A component with similar chromatographic properties was detected in the urine and feces of intermolt crabs (McCarthy and Skinner, 1979); this unidentified metabolite probably represents an excretion product of ecdysteroids.

Metabolic fate of ecdysone in various tissues

The change in the total radioactivity (Figs. 1A, B) and the relative amounts of the several ecdysone metabolites (Tables I and II) were determined in several

Table II

Ecdysone and its metabolites in LBA crabs. Symbols are as in Table I.

Tissue	Hr after injection	Component (% radioactivity)						
		NR	Polar conjugate of			Free ecdysteroids		
			20,26-OH	β	α	20,26-OH	β	α
Serum	1	—	—	—	—	1	56	43
	4	—	—	—	2	13	79	6
	9	—	—	—	—	19	78	3
	24	—	—	—	—	17	81	2
Epidermis	1	—	—	—	—	—	57	43
	4	—	—	—	6	9	73	12
	9	—	—	11	4	21	58	6
	24	—	—	11	—	15	72	2
Carcass	1	—	—	—	—	5	44	51
	4	—	—	—	—	3	65	32
	9	—	—	—	4	16	66	14
	24	—	—	—	—	24	76	—
Midgut gland	1	—	—	12	85	—	—	3
	4	—	1	26	62	—	—	11
	9	—	—	31	47	4	5	13
	24	—	4	43	21	8	14	10
Hindgut	1	22	—	—	—	—	42	36
	4	15	4	—	9	3	57	11
	9	28	6	—	7	14	42	3
	24	5	9	—	6	16	60	3
Total crab	1	—	—	2	12	2	43	41
	4	—	—	3	8	6	63	20
	9	—	—	5	11	15	59	10
	24	—	1	17	8	16	53	5

tissues at various times after injection of [^{3}H]ecdysone. The radioactivity present as each metabolite (cpm/g tissue) was also calculated (Figs. 2, 3).

Serum. The rate of loss of total radioactivity from the serum is rapidly accelerated by the autotomy of partially regenerated limbs. In crabs in stage D_0, radioactivity is rapidly lost for the first 4 hr ($k = 0.10$/hr), but the loss slows greatly during the remaining 20 hr of the experimental period ($k = 0.004$/hr). In crabs whose limb regenerates were re-autotomized at the time of injection, the rate of loss of radioactivity is rapid throughout the experimental period ($k = 0.04$/hr) (Fig. 1).

20-Hydroxyecdysone is the primary (> 80%) metabolite of ecdysone in both sets of crabs; however, the rate of loss of 20-hydroxyecdysone undergoes a twenty-fold increase from $k = 0.002$/hr for normal crabs in stage D_0 to $k = 0.04$/hr within 4 hr after LBA (Figs. 2A, 3A). The rate of loss of [^{3}H]20-hydroxyecdysone following LBA is approximately the same as the rate of decrease of serum ecdysteroid titers following LBA ($k = 0.046$/hr; McCarthy and Skinner, 1977b).

Epidermis. The loss of radioactivity from epidermis of crabs in stage D_0 has two kinetic components: a rapid loss initially ($k = 0.10$/hr), followed by a much

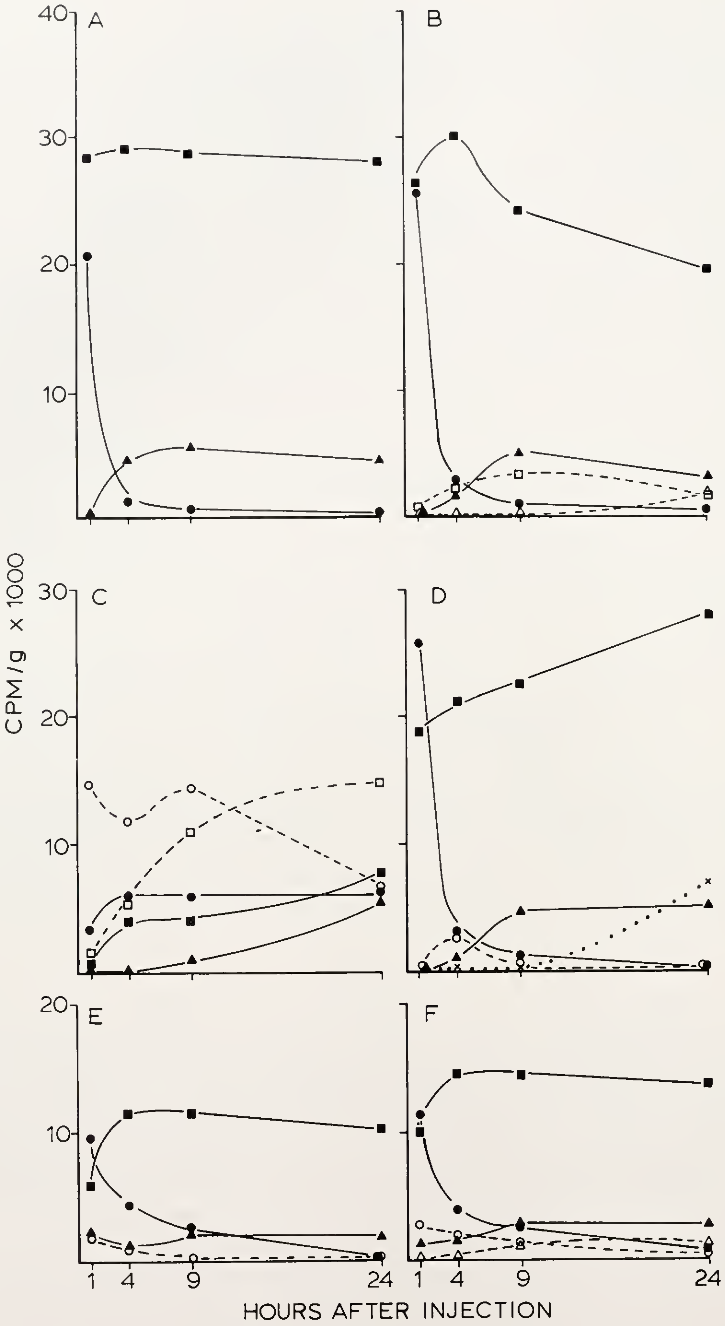

FIGURE 2. Concentration of [^{3}H]ecdysone and its metabolites in normal crabs in stage D_0. Free ecdysteroids are indicated by filled symbols and solid lines; conjugates of each ecdysteroid are indicated by open symbols and dotted lines: ecdysone (closed circles) and its conjugate (open circles); 20-hydroxyecdysone (closed boxes) and its conjugate (open boxes); 20,26-dihydroxyecdysone (closed triangles) and its conjugate (open triangles). The very polar metabolite which is not retained on the column even at 20% methanol is indicated by the crosses with dotted line. (A) serum; (B) epidermis; (C) midgut gland; (D) hindgut; (E) carcass; and (F) the total crab.

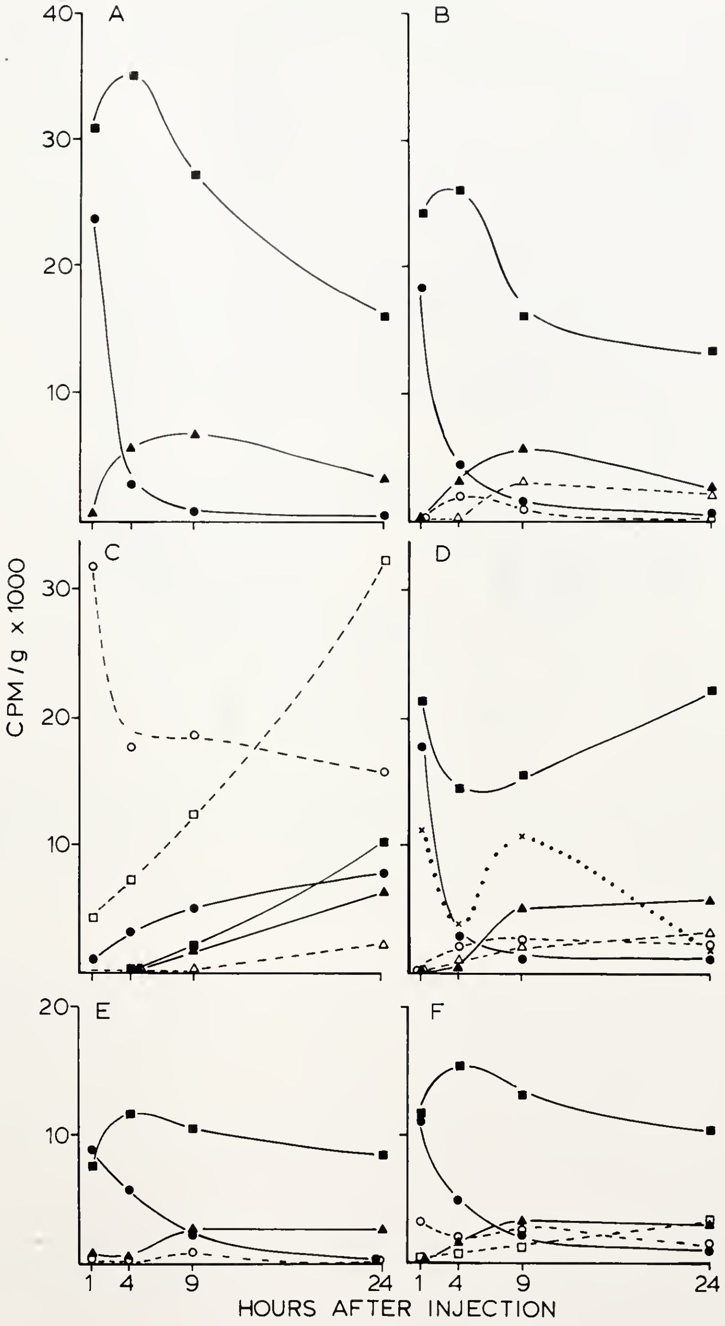

FIGURE 3. Concentration of ecdysone and its metabolites in LBA crabs. Legend same as in Figure 2.

slower loss during the remaining 20 hr (k = 0.02/hr). Following LBA, total radioactivity is lost at a relatively constant rate over the entire 24-hr period (k = 0.04/hr) (Fig. 1).

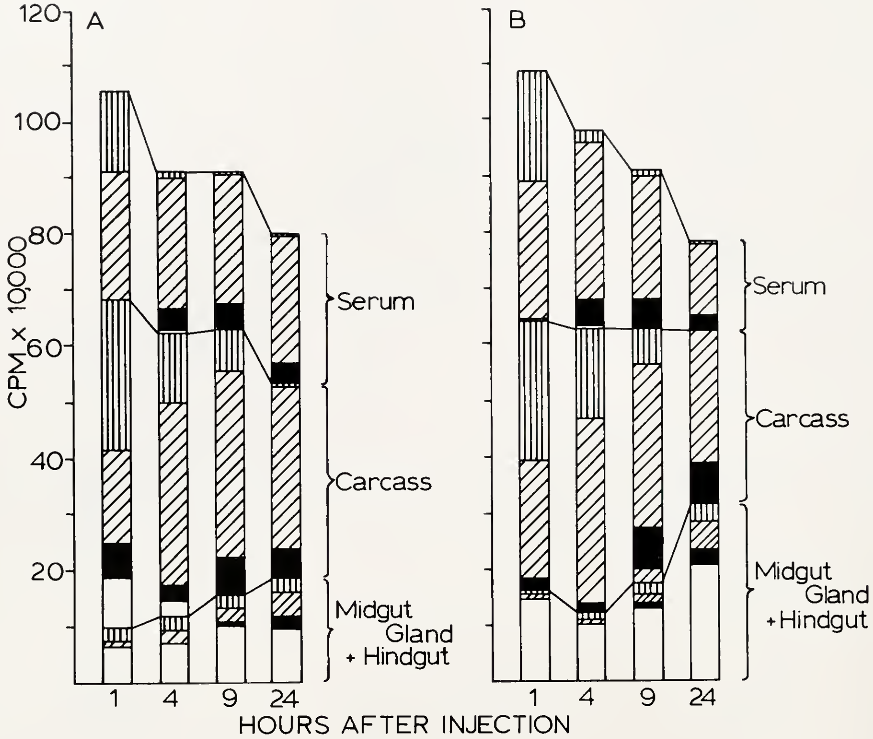

FIGURE 4. Pattern of change of radiolabeled metabolites in (A) normal crabs in stage D_0 and (B) LBA crabs. Changes in the total radioactivity in serum, carcass and midgut gland-hindgut over a 24-hr period are indicated by the diagonal lines between the bars for each time point. The amount of the various metabolites in each tissue at each time point is indicated: ecdysone (bars with vertical lines); 20-hydroxyecdysone (bars with diagonal lines); 20,26-dihydroxyecdysone (filled bars); total polar conjugates (empty bars).

20-Hydroxyecdysone is the primary metabolite of ecdysone; however, beginning at 4 hr, further hydroxylation to 20,36-dihydroxyecdysone becomes significant. The rate of loss of 20-hydroxyecdysone is slightly accelerated following LBA from k = 0.02/hr to 0.03/hr (Figs. 2B, 3B).

Carcass. The rate of loss of total radioactivity from the carcass is relatively unaffected by the re-autotomy of partial regenerates; the rate coefficients are k = 0.020/hr and 0.025/hr for normal crabs in stage D_0 and following LBA, respectively (Fig. 1).

20-Hydroxyecdysone and 20,26-dihyroxyecdysone are the primary metabolites observed in the carcass; the relative proportions of these metabolites increase steadily over the experimental period. The rate of loss of 20-hydroxyecdysone from the carcass more than doubles from k = 0.006/hr for normal crabs in stage D_0 to k = 0.014/hr within a few hours after re-autotomy (Figs. 2E, 3E).

Midgut gland. The major metabolites present in the mid-gut gland are polar conjugates of the ecdysteroids. The conjugate of ecdysone is the most abundant metabolite shortly after injection; however, as the concentration of this metabolite decreases, the amount of the conjugate of 20-hydroxyecdysone increases rapidly. In crabs in stage D_0, the amount of polar conjugates levels off toward the end of the experimental period, but following LBA, the concentration (cpm/g) of polar conjugates continues to increase so that at 24 hr, more than twice as much of these polar inactivation products are present in crabs from which limb regenerates were re-autotomized at the time of injection compared to normal crabs in stage D_0 (Figs. 2C, 3C). The elevated amount of these polar products is probably related to an increased rate of loss of ecdysteroids following re-autotomy.

Hindgut. 20-Hydroxyecdysone is the primary metabolite detected in the hindgut and its concentration increases fairly rapidly, especially in the latter portion of the experimental period. The hindgut also contained a metabolite not observed in any other tissue. This very polar metabolite was observed in fairly low amounts at the end of the experimental period in crabs in stage D_0, but appeared earlier and in larger amounts following LBA (Figs. 2D, 3D). Since this metabolite was previously detected only in feces of intermolt crabs (McCarthy and Skinner, 1979), the feces contained in the hindgut of the crabs of stage D_0 may be the source of this metabolite.

Metabolic fate of ecdysone in the whole crab

In crabs in stage D_0, the overall pattern of ecdysone metabolism is characterized by: metabolism of ecdysone primarily to 20-hydroxyecdysone, with further hydroxylation to 20,26-dihydroxyecdysone occurring to a limited extent; and catabolism of free ecdysteroids to polar conjugates.

The rate of loss of 20-hydroxyecdysone from crabs in stage D_0 is very slow ($k = 0.002/\text{hr}$); however, the rate of loss undergoes a tenfold acceleration within hours after LBA ($k = 0.02/\text{hr}$; Figs. 2F, 3F). The increased rate of loss of 20-hydroxyecdysone from LBA crabs is associated with an increase in the relative proportion and total amount of the polar conjugate of 20-hydroxyecdysone (Fig. 4) accumulated in the midgut gland, suggesting that the enhanced elimination of the active hormone is accomplished through an increased rate of catabolism to polar inactivation products.

Discussion

Metabolism of ecdysone in land crabs appears to procede primarily through: hydroxylation of the sidechain at C-20, then C-26 to form 20-hydroxyecdysone (50–80%) and 20,26-dihydroxyecdysone (5–20%), respectively; and conjugation of the free ecdysteroids to form glycosides. These basic pathways were observed in animals in the intermolt stage (stage C_4; McCarthy and Skinner, 1979), early premolt (stage D_0), as well as the later stages of proecdysis (stages D_1–D_3, McCarthy, in preparation). The metabolic fate of ecdysteroids does change at the various stages of the intermolt cycle with respect to: the rate of elimination or turnover of 20-hydroxyecdysone; and the amount of catabolism of free ecdysteroids to their polar conjugates.

As the animal enters into proecdysis the rate of turnover of 20-hydroxyecdysone decreases about three-fold and the amount of further metabolism to 20,26-dihydroxyecdysone and catabolism to polar conjugates also decrease. This inverse relationship between the rate of catabolism and excretion of ecdysteroids and the endogenous ecdysteroid titer during the period of hormone increase has also been observed in several insects (Koolman, Hoffmann and Dreyer, 1975; Hikino, Ohizumi and Takemoto, 1975; Feyereisen, Lagueux and Hoffmann, 1976; Weinheimer and Romer, 1977) and may be a regulatory mechanism common to arthropods.

The molt-related changes in the rates of catabolism and elimination of ecdysteroids appear, then, to contribute significantly to the observed increases in hormone titers during proecdysis; however, besides these long-term changes in the rate of elimination, the mechanisms regulating this elimination are capable of undergoing very rapid changes to bring about dramatic decreases in the endogenous hormone level. This rapid increase in the rate of elimination was observed in *G. lateralis* in late stage D_3. The rate coefficient for the elimination of 20-hydroxyecdysone increased from $k = 0.006/\text{hr}$ to 0.05/hr at 24 to 36 hr before ecdysis to reduce the serum ecdysteroid titer from 100 to 15 ng/ml during the last day before ecdysis (McCarthy, in preparation).

An even more rapid change in the rate of elimination occurs following re-autotomy of partially regenerated limbs (LBA) in stage D_0. The rate of loss of 20-hydroxyecdysone from the whole crab increased ten-fold within hours of LBA (Figs. 2F, 3F). The rate of loss from the serum increased twenty-fold, while the loss from the carcass (serum-free) only doubled following LBA. This relationship suggests that ecdysteroids destined for excretion are drawn from the serum pool rather than directly from an intracellular compartment. The ecdysteroids that are rapidly lost just prior to ecdysis also appear to be drawn preferentially from the serum compartment (McCarthy, in preparation).

Although the increased rate of loss of radiolabeled 20-hydroxyecdysone from the serum following LBA ($k = 0.04/\text{hr}$) is sufficiently rapid to account for the observed decline in serum ecdysteroid titers ($k = 0.046/\text{hr}$; McCarthy and Skinner, 1977b), the nature of the regulatory mechanism(s) responsible for this enhanced rate of elimination have yet to be elucidated. Whatever the mechanism, it is clearly not regulated by nervous or neurosecretory factors originating in the eyestalks. Even when animals are deprived of eyestalks, the source of the postulated molt-inhibitory hormone, re-autotomy of partial regenerates in stage D_0 elicits the same responses as in crabs with intact eyestalks: serum ecdysteroid titers decrease very rapidly, proecdysis is temporarily suspended, and a secondary proecdysis is then initiated (McCarthy and Skinner, 1977b).

The stimulus for the increased rate of elimination may be a disruption of normal proprioceptive input. Re-autotomy severs the proprioceptive signals from the affected limb or limb regenerate; the rapid loss of ecdysteroid immediately prior to ecdysis may likewise be related to the detachment of the apodemes of muscles from the fragile exoskeleton during the preparation for ecdysis. Although there is no direct evidence for the role of proprioception on ecdysteroid metabolism, loss of proprioceptive input in larvae of the insect *Galleria* resulting either from sectioning the ventral nerve cord or from immobilization of larvae within glass tubes delays pupal apolysis. This inhibition is mediated by the subesophogeal ganglia apparently by direct neural connection to a target organ (Edwards, 1966; Alexander, 1970). A direct neural inhibition of the prothoracic gland was suggested, but the data are not inconsistent with an enhanced rate of elimination of the molting hormone.

Although many aspects concerning the nature of the regulatory mechanisms responsible for the interruption and re-initiation of proecdysis following limb autotomy in stage D_0 have yet to be resolved, the present study has: confirmed the rapid loss in serum ecdysteroids following LBA; demonstrated that this loss results from a rapid enhancement in the rate of catabolism and elimination of ecdysteroids from the crab; and demonstrated that whatever the unidentified mechanism regulating this response, its primary mode of operation is to rapidly and directly affect the metabolism of the hormones responsible for regulating the molting process. These observations, as well as our pervious observations of the effect of eyestalk removal on serum ecdysteroid titers, limb regeneration and gastrolith deposition (McCarthy and Skinner, 1977a, b), make it clear that the regulation of the crustacean intermolt cycle is more complex and multi-faceted than suggested by the classic paradigm of antagonistic interactions between the eyestalk's molt-inhibitory hormone and the release of ecdysone from the Y-organ (Passano, 1960).

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SUMMARY

1. The metabolic fate of injected [^{3}H]ecdysone was examined in crabs in premolt stage D_0 and in crabs at this stage whose proecdysis was interrupted by re-autotomy of partially regenerated limbs at the time of injection.

2. In both sets of crabs, 20-hydroxyecdysone was the principal metabolite, with further hydroxylation to 20,26-dihydroxyecdysone and the formation of polar conjugates (primarily in the midgut gland) occurring to a limited extent.

3. The rate of elimination of 20-hydroxyecdysone from normal crabs in stage D_0 is slow ($k = 0.002$/hr). Within a few hours after re-autotomy of partial regenerates from crabs in stage D_0, the rate of loss of 20-hydroxyecdysone from the whole crab increases by tenfold, while the loss from the serum accelerates twentyfold.

4. The increased rate of loss of 20-hydroxyecdysone from the re-autotomized crabs is associated with an increased amount of catabolism to polar inactivation products. The increase in the rate of elimination of ecdysteroids is sufficient to account for the observed decrease in endogenous ecdysteroid titers when proecdysis is interrupted by this re-autotomy.

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THE BIOLOGY OF *AGLAOPHAMUS NEOTENUS* (POLYCHAETA: NEPHTYIDAE), A NEW SPECIES FROM MAINE AND CANADA[1]

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The biology of the family Nephtyidae Grube, 1850, is poorly understood. Most of the literature deals with taxonomic aspects. Hartman (1950) provides a review of the morphological and taxonomic features of the family, and recognizes three genera, *Aglaophamus, Micronephthys,* and *Nephtys,* as does Pettibone (1963). Fauchald (1968 and 1977) adds a fourth genus, *Inermonephtys.* Hartman points out that due to many simliar structural characters the family has been considered a single genus by Cuvier (1817), Savigny (1820), Audonin and Milne-Edwards (1834), and Chamberlin (1919). Fauchald (1968) demonstrates that the genera *Nephtys* Cuvier, 1817, *Aglaophamus* Kinberg, 1866, and *Micronephthys* Friedrich, 1939, are separated by well-established morphological characteristics, especially the nature of the branchae or interramal cirri which may be involute, recurved, or absent as described by Hartman (1950). In recognizing the new genus *Inermonephtys,* Fauchald (1968) emphasizes the nature of interramal cirri, proboscidal papillation, and antennae to separate the four genera of Nephtyidae. Other diagnostic features used by Fauchald include aspects of the jaw, nuchal processes, acicular tip, and parapodial setation. General features of the genus *Aglaophamus* are given by Hartman (1950) and more recently by Fauchald (1968 and 1977) who recognizes 45 species.

The first description of nephtyid larvae was by Claparède and Metschnikow (1869). Since then brief descriptions of planktonic forms have been made by Fewekes (1883), Haecker (1896), Leschke (1903). Gravely (1908), McIntosh (1908), Fuchs (1911), D. P. Wilson (1936), Smidt (1944), Thorson (1946), and Rasmussen (1973). These forms have all been attributed to two or three species of *Nephtys.* No larval stages have been reported for *Aglaophamus* or *Micronephthys.* There are no recorded observations of nephtyid metamorphosis from the planktonic laravl stage to the benthic juvenile. There appears to be no record of a complete life history of any species of nephtyid from egg to adult. The annual gametogenic cycles for *Nephtys hombergii* and *N. caeca* are described by Olive (1978).

Information of the feeding habits of nephtyids is limited. Mileikovsky (1959) demonstrated the pelagic larvae of *Nephtys ciliata* are predatory on both bivalve and gastropod larvae. Blegvad (1914) stated that adult members of this family feed mainly on smaller worms, molluscs, and crustaceans. In two cases, Hunt (1925) found remains of spionid worms in *Nephtys hombergii.* Pettibone (1963) wrote that nephtyids are predatory in their feeding, as did Mare (1942), and Clark (1962). Mare found that worms seldom had gut contents when examined, but if material was present it consisted of the remains of other polychaetes. Day (1967) pointed out that nephtyids were generally thought to be carnivores, but doubts had arisen after studies of dense populations. Sanders (1958, 1960) considered *Nephtys incisa* a nonselective deposit feeder. Evidence from the present study shows *Aglaophamus neotenus* is a nonselective omnivore, feeding on sediment.

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An undescribed species of *Aglaophamus* was first observed by the author in 1966 near the University of Maine's marine laboratory in Walpole, Maine. A dense population of animals was located off Wentworth Point in the Damariscotta River. The population extended from the intertidal zone to a depth of about 10 m. The sediment was a soft, silty mud. Adult animals were small (5 to 12 mm) and reproducing. Members of this species were later found at several other locations along the Maine coast including Montsweag Bay on the Sheepscot River, and the Orland River tributary to Penobscot Bay. Specimens were also sent to me by Dr. M. J. Dadswell of the Huntsman Marine Laboratory, which were collected from the St. John Estuary, Canada and tentatively identified as *Micronephthys* sp. They proved to be specimens of *Aglaophamus neotenus*, extending the range into Canada.

This paper describes the new species, its life history and biology.

MATERIALS AND METHODS

Subtidal samples of adult specimens of *A. neotenus* were taken with an Ekman grab having an opening of 225 cm^2. Animals were separated by washing the sediment through a No. 60 stainless steel sieve (0.250 mm mesh). Juveniles were collected subtidally at Wentworth Point by skimming the upper 1 to 2 cm of bottom with a metal scoop. This sediment was washed through a No. 140 stainless steel sieve (0.105 mm mesh). Planktonic larval forms were obtained by towing a No. 20 plankton net (0.076 mm) along the edge of the dock at Wentworth Point or from a boat near other collecting sites. Salinity and temperature measurements were made using a Beckman portable induction salinometer.

Living material was kept in controlled temperature cabinets with temperatures approximating those of the water at the time of collection. Adults and sediments retained by sieves were placed in 2-liter Pyrex culture dishes. Some juveniles and plankton isolates of larave were kept in Boveri dishes. Others were placed in 100-ml Pyrex funnels which had stems removed and bases melted closed. A small amount of sediment was placed in the bottom of each funnel, then sea water, and larvae or juveniles were added. The funnel culture method facilitated observation of swimming or burrowing. Some adults were kept in an aquarium with sediment from Wentworth Point.

Larval stages raised in the laboratory were lecithotrophic and required no food. Feeding experiments with adults gave negative results and will be discussed below. After washing, all glassware was rinsed once with concentrated HCl and three times with distilled water. Sea water was collected from the dock at Wentworth Point and filtered through Millipore glass fiber prefilters (Type AP20) before use.

In artificial fertilization experiments, gravid males and females were teased open with fine forceps in separate 500-ml Pyrex fingerbowls containing filtered sea water. Eggs were washed through a 0.250 mm nylon mesh screen into clean fingerbowls and allowed to settle. The supernatant was decanted and fresh filtered sea water added. This was repeated until most detrital and coelomic material was removed. Ten drops of "sperm water" were added from a dish containing male worms. Dishes containing eggs were held at room temperature until cleavage was observed. Cultures were then kept at 10° C.

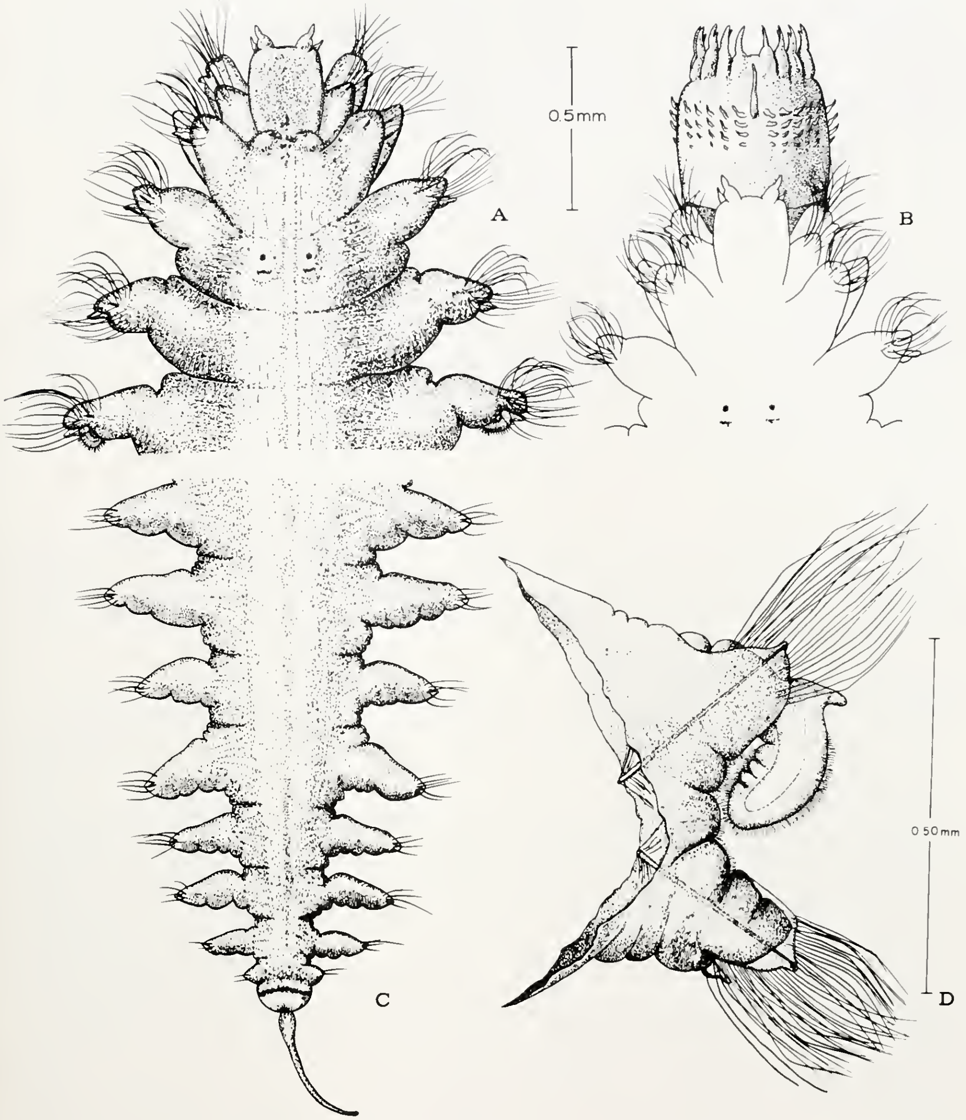

FIGURE 1. Adult specimen of *Aglaophamus neotenus*, new species: A, dorsal view of anterior end; B, view of anterior end with proboscis everted; C, dorsal view of posterior segments; D, anterior view of left, tenth, parapodial lobe.

Laboratory reared larval forms were cultured in 500-ml Erlenmeyer flasks at 10° C. Culture water was changed every second day by washing through a fine nylon mesh screen. Larvae retained on the screen were quickly and gently back-washed into fresh flasks with filtered sea water.

Hanging drop preparations were used to observe living gametes, cleaving eggs, larvae and juveniles. Glycerin mounts of preserved adults were studied to describe anatomical features. Living sperm were prepared as described by Franzen (1956), using Janus green B and neutral red vital stains.

Specimens were relaxed for study in a solution of propylene phenoxytol in sea water. A 0.15% solution was used for adults but was diluted approximately one-half for use with larvae. Worms were preserved in Kahles' solution and transferred to 70% ethyl alcohol for storage. Histological preparations included paraffin embedding, sectioning at 8 μ, and staining with hematoxylin and fast green.

Systematic Treatment and Results

Aglaophamus neotenus, new species

Type material

Holotype: National Museum of Natural History, Smithsonian Institution, USNM No. 47165, 1970, Gender female, 32 setigers, 10.3 mm long, 1.3 mm wide.

Paratypes (28): National Museum of Natural History, Smithsonian Institution, USNM No. 47166, 1970.

Material examined

The description is based on the holotype, paratypes, and specimens in the author's collection from Maine (Damariscotta River, Sheepscot River, and Orland River) and from Canada (specimens sent to the author by Dr. J. M. Dadswell, collected from the St. John Estuary).

Description

Adults very small, ranging from 5 to 12 mm long, 0.5 to 1.7 mm wide and having up to 32 segments.

Anterior end of body is blunt and narrow, body gradually widens in middle region, then narrows to the pygidium which carries an anal cirrus (Fig. 1C).

Prostomium rectangular, bears pair of anterodorsal antennae and pair of subterminal antennae (Fig. 1A). Antenna fleshy with bulbous base tapering to fine tip. Occasionally (in about 10% of worms examined) one or both ventrolateral antennae have a small lateral extension or bifid aspect. Nuchal organs arise dorsal, just posterior to prostomium, but are not always visible due to retraction, especially with preservation. Digitiform nuchal processes described by Fauchald (1968) for the genus *Inermonephtys* are lacking.

Eyes visible dorsally on setiger three, dark red in color, subepidermal; each has a round, granular pigment spot, with a crescent of fine pigment granules posterior to it.

Parapodia small, biramous, with those on first and last setiger being reduced. Small, dorsal, notopodial cirri begin on setiger two, are replaced by branchae or interramal cirri from setiger five or six through 15 or 20, but occur again through last setiger. The neuropodial, post-setal lobe, produced ventrally on the parapodium, carries the ventral cirrus on all neuropodia. Ventral cirri are reduced posteriorly.

Branchae or interramal cirri slightly involute, heavily ciliated, and carry an accessory crirrus on the outer side of their nonbulbous base (Fig. 1D).

Each noto- and neuropodium bears an aciculum with fine, recurved tip (Fig. 2D); acicular lobes are pointed. Anterior setigers have smooth capillary, preacicular barred capillary, and postacicular spinous setae. Barred setae number

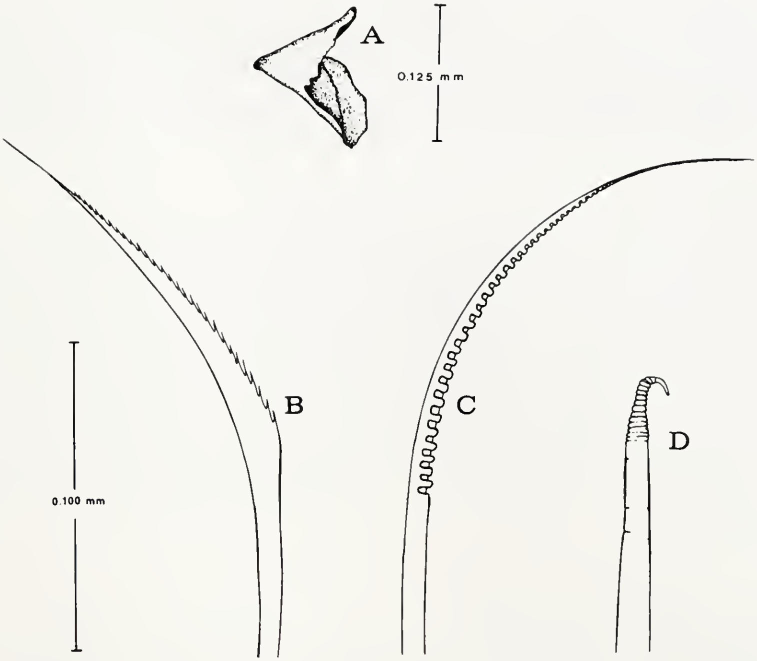

FIGURE 2. Adult specimen of *Aglaophamus neotenus*, new species: A, right jaw lying on its anterior surface, B. postacicular spinous setae, C, preacicular barred capillary setae; D, aciculum.

six to eight per fascicle and are replaced by smooth capillary setae at about setiger 20. Spinous setae are replaced by smooth capillaries over last few posterior setigers. Ornamentation of barred capillaries is on the outer one third of shaft (Fig. 2C). Spinous setae have blade-like edges subdistally, bearing fine spinets (Fig. 2B).

Pharyngeal region occupies setigers two through eight, is swollen due to retracted proboscis, and provides slightly upward-curving appearance to worm.

Proboscis cylindrical, with distal row of 16 or 18 papillae with 14 to 16 longitudinal rows of fine subterminal papillae behind distal papillae; five to six fine, pointed papillae occupy each subterminal row. A single, long, mid-dorsal subterminal papilla is present. Proximal surface of proboscis is free of ornamentation (Fig. 1B).

Jaws very small, about 125 μm on longest axis, color golden brown, base roughly triangular; an inner supportive ridge appears to be present, but jaw detail is difficult to determine due to small size (Fig. 2A).

Body coloration in living worms varies from creamy white to pink or light orange; females more pink or orange than males. Dorsally, prostomium is yellow; dark red, sometimes almost black pigment granules are scattered over its surface. A dark red pigment spot is located on the distal and slightly ventral end of the prostomium. Prostomial pigmentation can vary somewhat between individuals. Paired, dark red, pigment spots, located dorsally on the pygidium may be "pygidial eyespots". The distal end of the pygidium is yellow. Fading of pigmentation occurs to varying degrees with preservation.

Distribution

From Maine, Damariscotta River, Sheepscot River, and Orland River; and from Canada, Kennebecasis River, Saint John Estuary, and Minas Basin, Nova Scotia (personal communication from Dr. M. J. Dadswell on Canadian distribution).

Remarks

Criteria used by Fauchald (1968, 1977) to separate the four genera of Nephtyidae can be applied to determine the proper genus for this new species. The presence of interramal cirri prevents describing this animal as *Micronephthys*, which lacks these organs. Despite this animal's very small size when adult, with care, the slight but definite involution of the interramal cirri can be seen. This condition, and the presence of recurved, acicula tips, negate choosing Nephtys which has recurved interamal cirri and straight acicula tips. Two pairs of antennae, ornamentation of the proboscis, and the lack of eversible digitiform, nucal processes prevent considering this species as *Inermonephtys* and justify placing it in *Aglaophamus*.

Ecology and behavior of adults

Aglaophamus neotenus lives in a polyhaline environment. Salinities at Wentworth Point in the Damariscotta River seldom drop below 30‰, those in the Orland River, and Montsweag Bay in the Sheepscot River, may decrease to about 19‰ in spring. Temperatures at all three locations are typical for Maine waters. All reach 0° C or less during the winter months, and as high as 17° C in late summer. Sediments associated with *A. neotenus* are mixtures of fine silt, clay, and sand grains, and include a large amount of organic material. The Orland River is unusual in that its sediment is composed of a very high percentage of wood chips. All three sites in Maine are sheltered from the open sea but exposed to tidal currents.

Adults from Wentworth Point and Montsweag Bay have a small ciliate associated with them. It appears to be of the order Peritricha and can be seen gliding over the inner surfaces of the parapodial rami. Hyman (1940) states that peritrichs can be epizotic and possibly ectoparasitic. The peritrichs on *A. neotenus* may be utilizing as food, particulate matter carried by the interramal currents. These ciliates do not appear to harm *A. neotenus* and the relationship may be commensal in favor of the ciliate.

In an attempt to determine the type of food eaten by *A. neotenus,* starved individuals were placed in dishes containing fresh *Mytilus edulis* tissue, dried liver powder, clumps of the brown diatom, *Phaeodactylum tricornutum,* and a green filamentous alga from the sediment surface at Wentworth Point, all materials which had been useful foods for other polychaetes in our laboratory. There was no response to these food materials.

The diet of *A. neotenus* was revealed when freshly collected specimens were observed voiding fecal pellets. The fusiform shaped pellets were encased in mucus, and connected to other pellets by a fine mucus string. Single pellets with a mucus string at one end were also observed. Pellets contained a mixture of materials including several species of benthic diatoms, empty copepod exoskeletons, unidentified organic material, and fine sand grains.

Aglaophamus neotenus moves in the usual manner of other nephtyids, burrowing actively and swimming when disturbed. Animals kept in an aquarium containing sediment were observed making burrows which opened to the surface. Movement in the sediment was active, and some swimming occurred when undisturbed. On occasion, worms were seen swimming 10 cm above the sediment.

Reproduction

Gravid females are easily distinguished by their pink or orange color and the presence of eggs densely packed in the coelom. The pink or orange color diminishes during the spawning period and the worms become whiter in color. Eggs extend into the parapodial rami, and are even observed in the coelomic space of an everted proboscis. In maturing females, eggs are fewer in number and only visible from setiger eight through the last setiger. Size of eggs in the coelom varies with maturity of the worm. The largest eggs measured inside preserved and cleared (glycerin mount) specimens, or histologically sectioned and stained females, were about 60 to 70 μm in diameter.

Spawning males are creamy white or yellow in color. Their coelomic spaces, including the parapodial rami, are slightly swollen with a fine, granular appearing material.

The Wentworth Point population had about twice as many males as females. Females were slightly larger than males. Further study is needed on sex ratios in this and other populations of *A. neotenus*. Preliminary histological studies showed a pre-spawning cytology for gravid adults to be similar to that of *Nephtys hombergii* and *N. caeca* as described by Olive (1978), but more detailed work must be done to determine precise differences or similarities.

Males and females were observed to spawn as they swam across the bottom of a culture dish. Males emitted a jet of milky fluid from the anus. The fluid took the form of a fine thread which, in a matter of seconds, dispersed into a milky cloud of sperm mixed with what appeared to be coelomic fluid. Eggs were released from females in a similar manner. Eggs moved down the coelomic cavity to the pygidial area and emerged from the anus in a fine string. The fluid accompanying eggs was more viscous than that with the sperm, and tended to remain visible with eggs as they settled to the bottom of the dish. In both sexes gametes were observed passing into the anal tract through a break in the coelomic wall. Individual worms known to have spawned, still contained gametes for over a month after initial spawning was observed.

Although adults from Wentworth Point contain gametes through the year, they are most obviously gravid in late winter and early spring. Spawning occurs in late March, April, and possibly early May (based on conditions of adults and the presence of planktonic stages in surrounding waters). Worms spawned "naturally" in the laboratory through April and May under the same temperature and salinity conditions found at that time off Wentworth Point.

Embryology

Eggs when released in the laboratory, are 45 to 80 μm in widest diameter and average 70 μm. They resemble flattened discs, much like human erythrocytes. Each egg contains a large granular nucleus with an accompanying nucleolus. The cytoplasm is granular, opaque, and white in color. Upon contact with sea water, eggs become spherical within 15 min and often show membrane invagination and clear bubble-like swellings at their surfaces.

Sperm are of the primitive metazoan type described by Franzen (1956). The head and mid-piece are 5 μm and the tail is 70 μm long. The tail ends in a fine hair-like tip. The spherical head contains a darkly staining nucleus, and there are four, round mitochondrial spheres in the mid-piece. A small, nipple-like acrosome is carried on the distal end of the head.

A detailed study of cleavage and cell-lineage was not done, but the general pattern of cleavage was observed, and appears typical of other annelids (see E. B. Wilson, 1892, and Kume and Dan, 1968).

A fertilization membrane appears 1 hr after fertilization. In five hours, a two-cell stage with distinct polar bodies is observed. The four-cell stage is reached in 6 hr, the eight-cell stage an hour later. During the following 10 to 15 hr, 16- and 32-cell stages are seen.

Larval development

Names of larval stages are after Gravely (1909).

Protrochophore. Twenty-four hours after fertilization a dense, dark green embryo (reflected light) is present inside the fertilization membrane. The anterior end is somewhat conical, and is composed of large embryonic cells. A prototrochal band of fine, short, beating cilia surrounds the equator of the embryo. Rotation inside the fertilization membrane does not occur. During the next 24 hr the prototroch protrudes through the fertilization membrane, and the larva begins to swim. This stage is 90 μm in widest diameter.

Early trochophore. An early swimming trochophore is seen in about 48 hr (Fig. 3A). The fine cilia of the protrochophore have been replaced with a single, well developed band of long cilia. This prototroch divides the larvae into two hemispheres, the anterior one being smaller. An apical tuft of long cilia protrudes at the anterior end of the larva. A gut is not present but the center of the early trochophore is packed with dense material, darker than the greenish-brown cytoplasm of the surface cells, when seen by reflected light. Small, yellowish-green pigment vacuoles and granules are scattered over the surface of the larva. The early trochophore is 90- to 100-μm long.

Trochophore. During the next 7 days, the early trochophore develops into a more advanced trochophore form (Fig. 3B). The fertilization membrane is adsorbed and becomes part of the larval cuticle. The surface of the cuticle is scattered with yellowish-green pigment vacuoles and granules, as in the early trochophore. The apical tuft is lost, but fine sensory cilia are distributed over the head. The prototroch has developed two rows of cilia, an anterior band of long cilia and a posterior band of fine, short cilia. (Only two rows of cilia could be seen clearly, but a third may be present.) Occasionally, a long, heavy cilium is observed laterally on the prototroch. Early in the trochophore stage no distinct gut is visible but during the next 4 weeks the dense material at the center of the trochophore develops into a ciliated cavity filled with yellowish-green, yolky material. A ciliated mouth opens during this period slightly posterior to the prototroch. A fine band of cilia, the neurotroch, extends from the mouth to the posterior surface of the trochophore. On several occasions, what appeared to be an anal opening was seen, but its presence was not confirmed. Precisely when in development the anus opens was not determined, but it is present in the Metatrochophore I stage. The trochophore is about 100 μm long.

The above descriptions represent larval development through about 30 days to the trochophore stage. It was not possible to raise trochophores to the Meta-

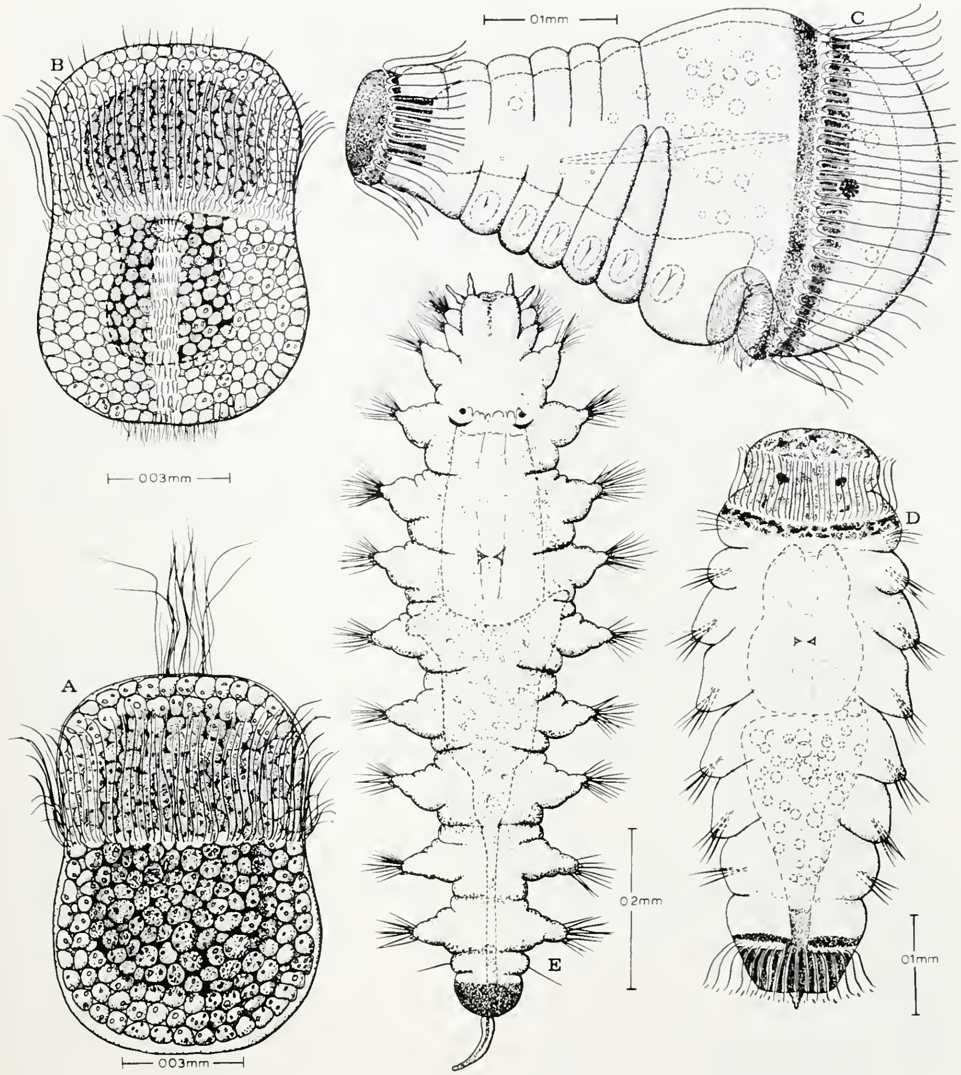

FIGURE 3. Larvae of *Aglaophamus neotenus*, new species: A, early trochophore; B, trochophore; C, Metatrochophore I with diatom present in gut; D, Metatrochophore II, E, juvenile.

trochophore I stage in the laboratory; however, Metatrochphore I and II stages were both collected from plankton tows and from sediment containing adult worms at Wentworth Point. These planktonic stages survived in the laboratory, and metamorphosed into juveniles whose characteristics were so like the adults as to dispel any doubt that they were young specimens of *Aglaophamus neotenus*.

Metatrochophore I. The simplest form of segment larvae, the first metatrochphore (Fig. 3C), appears in the plankton in May. When viewed laterally, the larvae are triangular in shape. Eyes are seen for the first time, a pair are lateral on the head, and slightly anterior to the prototroch. They are simple, round spots of red pigment granules. The prototroch is denser, but essentially the same as in the trochophore. A teleotroch has developed as a single band of long cilia around the pygidium. The neurotroch is no longer present. A ciliated anus now opens on the posterior end of the larvae. Fine sensory hairs are still scattered over the head. The mouth has enlarged and a pair of ciliated lips has appeared. Up to six segments have formed, and setal sacs are present ventrally; dorsal sacs develop slightly later. Reflected light shows dark, reddish-brown pigment on both sides of the prototroch and telotroch. The distal portion of the pygidium is also heavily pigmented with the same reddish-brown pigment. Yellow pigment is found with the reddish-brown pigment. A complete gut is present, and it contains brilliant, yellowish-green, yolky material. The anal end of the gut, when viewed by reflected light, is filled with opaque, white yolky material. On several occasions diatoms were seen inside the gut. The Metatrochophore I is about 450 μm long. Specimens of this stage were raised into juveniles in the laboratory.

Metatrochophore II. In late May and early June the second metatrochophore stage, Metatrochophore II, is found in the plankton. It is cylindrical, with distinct prostomial and pygidial areas separated by segmental grooves from the setigerous segments (Fig. 3D). Parapodia are now present, and carry barred and smooth capillary tipped setate on both neuropodia and notopodia. Acicula have developed, and rest in small acicular sacs on the tips of the parapodial rami. Eyes are present dorsally on the prostomium, and have clear lenses posterior to comma-shaped spots of red pigment. The prototroch, having lost a band of cilia, and the telotroch, remain as single bands of long cilia. No other ciliation is seen except around the ventral mouth and the anus. A muscular pharynx with a pair of brown jaws is present, but the proboscis has not formed. The gut tapers from pharynx to anus. It is still colored a bright greenish-yellow, but contains fewer yolky vacuoles. The opaque, white yolky material is still present at the anal end of the gut. A small anal cirrus is seen ventral to the anus. Dark, reddish-brown pigment still surrounds the prototroch, but is less dense and has more yellow pigment associated with it. The head is covered with large, irregular-shaped spots of yellowish-brown pigment interspersed with dark red pigment granules. The telotrochal, and pygidial areas are still darkly pigmented, but have more yellow pigment associated with them than in the Metatrochophore I. The Metatrochophore II stage grows from six to eight setigers during its planktonic life and is about 550 μm long.

Metamorphosis. In June the eight-setiger Metatrochophore II undergoes a brief swim-crawl stage, sheds its larval ciliation, and burrows into the sediment. This is the beginning of metamorphosis. Approximately two weeks later, most of the larval characteristics are lost and a ten-setiger juvenile has developed.

Juvenile. The ten-setiger juvenile is about 800 μm long, it is essentially a minature adult (Fig. 3E). The body shape view dorsally is adult like, and gross morphology of the prostomium and pygidium resembles that of the adult, with antennae and an anal cirrus well developed. Eyes like the adults are present on setiger three. They may represent migration of prostomial eyes of the earlier Metatrochophore II posteriorly, as described by Fewkes (1883). Juvenile parapodia are well formed and carry ciliated, interramal cirri with accessory cirri. Ventral neuropodial cirri are present on all setigers. All adult setal types are seen in well developed fascicles, acicular sacs carry brown acicula. The retracted proboscis with a pair of triangular jaws can be seen through the translucent body wall and occupies setigers three through five. Dark pigment spots, pygidial eyespots are present on the pygidium. Pigmentation is otherwise transitional between the Metatrochophore II and mature adult.

Larval behavior

No larval stage showed either positive or negative phototaxis in the laboratory.

Locomotion was observed. The protrochophore is a feeble swimmer, but in the early trochophore, and trochophore stages, active swimming occurs with the larva spiraling around its longitudinal axis as it moves through the water. The Metatrochophore I is a strong swimmer, but spiraling was not observed in this stage. The Metatrochophore II is much slower in its swimming than previous stages, and often drops to the bottom to "test" the sediment in a swim-crawl like manner. Juveniles move actively through the sediment, and make mucus-lined burrows. The head of a juvenile often protrudes slightly from a burrow. Juveniles were not observed to swim above the sediment.

Feeding seems to occur fist in the Metatrochophore I, as diatoms were observed in the digestive tract of this stage (Fig. 3C). No evidence of actual digestion was seen except the presence of oil-like vacuoles in the digestive tracts of Metatrochophore I and II stages, which appeared to be from digested diatoms. The digestive tracts of juveniles were filled with a brown organic material, and they may be feeding on sediment, as do the adults.

DISCUSSION

Aglaophamus neotenus is an unusual member of its genus. The adult has a confusing mixture of what are normally considered larval and adult characters, as pointed out in a personal communication from Dr. Kristian Fauchald of the Allan Hancock Foundation. Larval characters are strong ciliation of the branchiae and the presence of pygidial eyes. Adult characters include the well developed proboscis and parapodial lobes. *Aglaophamus neotenus* appears to be the only member of its genus that reproduces at such a small size.

The retention of some larval characters as an adult, and the attainment of sexual maturity at a small size justify referring to *A. neotenus* as a neotenic species.

The fact that fecal pellets of *A. neotenus* contain several types of diatoms, the remains of copepods, and other sediment materials indicates it is a nonselective omnivore and feeds by ingesting sediment. This support Sanders (1958, 1960) who examined the gut contents of *Nephtys incisa* and concluded that it was a deposit feeder. Clark (1962) felt nephtyids were carnivores, and Mare (1952) found the remains of other polychaetes in the gut of a specimen of *Nephtys* sp. It now appears that there is a range from omnivore to carnivore in the Nephtyidae, with some feeding selectively and others nonselectively.

Comparing the development of *A. neotenus* with information given by previous workers reveals the general aspects of development in Nephtyidae. The early stages described by D. P. Wilson (1936) for *N. hombergii* closely parallel those of *A. neotenus.* Ciliation is the same in the trochophores of both species except that Wilson observed two small patches of cilia anterior to the prototroch and three rather than two bands in the prototroch. Pigmentation is generally the same in both species and part of the cuticle is formed by absorption of the fertilization membrane in both cases. The apical tuft is lost in *N. hombergii* at about the same time as in *A. neotenus.* Wilson observed a distinct anus in *N. hombergii* trochophores; this structure was unclear in *A. neotenus. Nephtys hombergii* has eggs which are 32 μ larger than *A. neotenus;* the trochophore in *N. hombergii* is 60 μ larger.

Fewkes (1883) describes a stage of *Nephtys* sp. which is significant, because it is transitional between the trochophore and Metatrochophore I stage. This stage was no longer spheroidal but had elongated to double the original hemispherical length. Little growth had occurred in the preoral lobe, rather, most elongation had taken place in the postoral area. Green coloration had appeared around the pole of the preoral lobe. Most of the cilia (early trochophore cilia) which were scattered over the external surface of the body had disappeared. The lower body hemisphere was described as becoming elongated and segmented but no definite parapodial structures had developed in the several body segments. The intestine had lengthened and its walls and those of the stomach were still green as in younger larvae. Fewkes illustrated this stage which showed a well developed prototroch and telotroch.

Metatrochophore I stages have been observed for the Nephtyidae by several authors: Fewkes (1883) and Haecker (1896)—*Nephtys* spp.; Leschke (1903) and Thorson (1946)—*Nephtys ciliata.* The Metatrochophore I of *A. neotenus* agrees with those described by these workers. Common to all are: a triangular shape when viewed laterally; ciliation restricted to a prototroch and a telotroch; dorsal eyes anterior to the prototroch; a distinct green, yolky gut, sometimes containing diatoms; and dark reddish-brown pigmentation on the pygidial area and at the bases of the telotroch and prototroch. The Metatrochophore I attributed to *N. ciliata* by Thorson is larger than that stage in *A. neotenus* by about 100 μ.

Metatrochophore II stages were described by the following authors: Claparède and Metschnikow (1869), Fewkes (1883), Gravely (1908), and McIntosh (1908) —*Nephtys* spp.; Hofker (1920), and Thorson (1956)—*N. caeca;* Smidt (1944) and Thorson (1946)—*N. ciliata;* and Thorson (1946)—*N. hombergii.* The general features in various descriptions given for the Metatrochophore II agree with my findings for *A. neotenus:* a body form which is more or less cylindrical with distinct prostomial and pygidial areas; dark red eyes present dorsally on the prostomium; well developed parapodial lobes carrying setal fascicles; preacicular barred setae; ciliation restricted to a telotroch and prototroch; a pharynx with jaws but lacking a proboscis; a dark green, yolky gut narrowing to an anus; an anal cirrus developing on the ventral surface of the pygidium; and dark pigment covering the pygidium and present to a lesser degree around the prototroch. Metatrochophore II stages attributed to the genus *Nephtys* are about 100 μ longer than this stage in *A. neotenus.*

There is not enough specific information given in the literature to make a key which would separate the various nephtyid stages described from larvae of *A. neotenus,* but possibly pigment patterns could be used to separate nephytid larvae. Fewkes (1883) used color patterns of the anal area to identify larvae of different stages of the same species of *Nephtys* sp. obtained from the plankton.

There is no previous report of swim-crawl or settling activities of nephytids in the literature, and juvenile stages have not been described.

Metamorphosis from the Metatrochophore II into juveniles has not been reported, although Fewkes (1883) discussed the migration of eyes from the prostomium to setiger four in Metatrochophore II stages of *Nephtys* sp. He felt that migration was caused by the forward growth of the head and anterior body segments.

Attempts to rear nephtyid larvae in the laboratory fail after about 30 days. Fuchs (1911), working with *N. hombergii,* raised larvae to the trochophore stage in about 14 days, after which no further development took place. All died within 30 days. The same was true for D. P. Wilson (1936) with *N. hombergii* and in my work with *Aglaophamus* sp. I do not think food is a limiting factor. The trochophores are densely packed with yolky material and a planktotrophic existence is unlikely in early stages. The presence of minute amounts of contaminants such as formalin, detergents and other organics are known to limit and even stop larval growth in many invertebrates. This may have been one factor which limited my success. Other factors such as the density of larvae in culture, light, temperature, and proper agitation may influence success or failure. As in any culture procedure, duplication of the natural environment is difficult and success may depend on the tolerance of a species to conditions imposed on it in the laboratory.

In this study Metatrochophore I stages were collected from the plankton and then reared to juveniles whose characteristics were so like the adults as to dispel any doubt that they were the same species. Metatrochophore II stages were also found in the sediment associated with the adult population, and also grew into juvenile specimens of *N. neotenus* in the laboratory. The challenge still remains to maintain nephtyid trochophore stages beyond 30 days, and raise them to Metatrochophore I stages which will then grow into juveniles and adults in the laboratory. This study nevertheless presents the most complete life history to date of any species in the family Nephtyidae.

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SUMMARY

1. Populations of a previously undescribed polychaete, *Aglaophamus neotenus,* were found in three polyhaline locations on the Maine coast. Specimens were also received from Canadian workers. Temperatures in the Maine locations ranged from 0° C in winter to 17° C in the summer.

2. Diagnostic characters of the adult include heavily ciliated, involute interramal cirri which bear accessory cirri, present from setiger 5, sometimes 6, through setigers 15 to 20; the presence of acicula with recurved tips, capillary tipped, barred and spinous setae, and the lack of furcate setae; two pairs of antennae on the prostomium; ornamentation of the proboscis; the lack of eversible digitiform nucal processes; and the presence of pygidial eyespots on a spherical pygidium which is ringed with red pigment.

3. Adults reproduced at a size smaller than was previously reported in the Nephtyidae. Spawning was observed in the laboratory. Sperm are primitive. The eggs are granular, white in color, and resemble human erythrocytes in shape. Eggs average 70 μ in widest diameter.

4. Cleavage was spiral and appeared similar to that of other polychaetes.

5. Larval stages included: protrochophore, early trochophore, trochophore, Metatrochophore I, Metatrochophore II, and an eight-setiger swim-crawl-stage. Metamorphosis of the swim-crawl stage resulted in a ten-setiger juvenile which was essentially adult in morphology.

6. Behavior of adults and larvae was observed. Adults burrowed actively

and swarm above the sediment even when undisturbed. Larvae swam vigorously in early stages but slowed down as the swim-crawl stage was approached. Juveniles burrowed actively through the sediment and made mucus-lined burrows.

7. The diet of *A. neotenus* was studied. Larvae are lecithotrophic through the trochophore stage but diatoms were observed in the gut of Metatrochophore I stages. Observation of gut contents indicated juveniles feed on sediment. The digestive tract of adults contained materials, revealed by the contents of fecal pellets, which demonstrated that they were nonselective omnivores, feeding on sediment.

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OXYGEN UPTAKE AND TRANSPORT IN THE PROSOBRANCH MOLLUSC *BUSYCON CANALICULATUM* (L.) II. INFLUENCE OF ACCLIMATION SALINITY AND TEMPERATURE

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At summer temperatures and in high salinity waters, the prosobranch mollusc *Busycon canaliculatum* (L.) utilizes an unusually large fraction of the oxygen in its blood, permitting a relatively high rate of oxidative metabolism (Mangum and Polites, 1980). Like most molluscs, the conch regulates physical and chemical conditions in the blood either very weakly or not at all. Still, the species is found in both polyhaline and euhaline waters from Cape Cod to Florida, where it may experience an annual temperature change in excess of 20° C and a salinity range of 18 to 35‰. This magnitude of environmental change should seriously perturb the performance of the oxygen transport system. At the mean annual temperature in the middle of the geographic range (10° C), the oxygen affinity of conch hemocyanin is four times higher ($P_{50} = 3$ mm Hg at pH 7.9 and 35‰ salinity) than at 22° C ($P_{50} = 12$ mm Hg under the same conditions) (Mangum and Lykkeboe, 1979). At 18‰ salinity, the cooperativity of oxygen binding sharply decreases, which could seriously impair oxygen uptake by lowering the oxygenation state of the blood at branchial P_{O_2}.

In addition to direct effects on the oxygenation of the carrier molecule, temperature and salinity may influence the respiratory properties of the blood indirectly, by means of effects on blood pH. In many animals blood pH increases at low temperature or low salinity, and this change could be especially important in *B. canaliculatum* because pH is an unusually strong and complex determinant of hemocyanin oxygenation (Mangum and Lykkeboe, 1979). In crustaceans, the changes in blood pH accompanying adaptation to low salinity waters oppose the respiratory effects of salt loss from the blood. While the salt loss lowers oxygen affinity, the concomitant pH increase raises oxygen affinity, and the net result is an oxygen transport system that works better under the particular combinations of environmental and behavioral conditions that accompany the migration upstream (Mangum and Towle, 1977). The changes in blood pH are intimately related to the regulation of salt and water content of intracellular and extracellular body fluids. In gastropods, regulation of blood salts is known only at very low salinities not within the range tolerated by neptuneids (Staaland, 1970). While cell volume appears to be regulated quite strongly (Staaland, 1970), the magnitude of the changes in free amino acids is conspicuously variable among different species (Schoffeneils and Gilles, 1972), which implicates the participation of other osmolytes that may have quite different effects on blood pH. Therefore, the net result of low salinity adaptation on the oxygen transport system cannot be predicted.

We have studied the effects of temperature and salinity an oxygen transport, with emphasis on the relationship to total oxygen uptake.

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METHODS AND MATERIALS

The measurements were made on animals collected throughout the year to minimize the difference between natural and experimental conditions. The animals were held at a particular combination of temperature and salinity for a minimum of 1 week, if the environmental change was small, and a maximum of 3 weeks.

The procedures used in collecting the respiratory data are described by Mangum and Polites (1980). Osmolality was measured with an Osmette freezing point osmometer, chloride with a Buchler-Cotlove chloridometer, ammonia by the phenol hypochlorite method (Solòrzano, 1969; Gravitz and Kleye, 1975) and total ninhydrin positive substances as described by Henry and Mangum (1980). The free amino acid composition of pedal muscle tissue was determined with a Technicron Auto amino acid analyzer, as described by DuPaul and Webb (1970). The tissue was excised, blotted dry, extracted with 80% ETOH for 2 days, and then dried at 60° C to constant weight.

After the profound disturbance accompanying changes of the medium or transfer to another vessel, conchs often take a very long time to emerge from their shells and resume the exchange of metabolites with the medium. Therefore, the measurements of ammonia and TPNS excretion were made in the running sea water systems of the laboratories of the Virginia Institute of Marine Science. Nitrogen excretion was first determined at high salinity (33‰) at the Wachapreague Laboratory by placing animals (starved for 1 week) in a 10-liter container of running sea water. After ascertaining that the spihon was extended, the tap was closed and the addition of nitrogen compounds to the medium was assayed for 1 hr. The animal was then transported to the Gloucester Point Laboratory, allowed to acclimate for 7 days to running 18‰ water, and the experiment was repeated.

The behavior pattern also seemed to influence the oxygen uptake measurements, in that the period required for emergence after disturbance was highly individual. Paired observations on the same animals acclimated to different salinities were made of oxygen uptake as well; in this instance the low salinity animals were held in very large (60-liter) containers of standing, aerated water.

Data analyses were performed according to Student's t test, for either paired or unpaired observations.

RESULTS

Behavior

The ambient temperature varied from 21 to 24° C during the experiments at high temperature. While the animals appeared to behave normally at temperatures lower than 23° C, they were conspicuously sluggish at 24° C, especially at low salinity, and considerable mortality occurred ($> 50\%$). The same pattern occurred in *B. carica*. This finding, although highly repeatable, seems somewhat anomalous in view of the geographic ranges of the two species, although local reports of "estivation" and offshore migration in mid-summer may explain the anomaly.

At 10° C locomotion in *B. canaliculatum* is reduced but not entirely absent, and the siphon is still extended, though less than at higher temperatures. Below that threshold, which delimits the abrupt transition between summer and winter conditions, conchs burrow fully into the sediment and retreat more than halfway

TABLE I

Effect of acclimation salinity and temperature on the osmotic and ionic properties of the blood in Busycon canaliculatum. *Mean ± s.e. (N).*

	6° C	10° C	22–24° C		
Osmolality milliosmoles					
Water	546	971	466		915
Blood	560 ± 1 (11)	977 ± 3 (7)	468 ± 1 (6)		918 ± 2 (11)
Chloride (mM)					
Water			271	535	554
Blood			257 ± 1 (8)	518 ± 2 (8)	529 ± 3 (9)
Blood pH (pedal)					
33–34‰	7.923 ± 0.024 (24)	7.945 ± 0.015 (38)	7.853 ± 0.009 (24)		
20‰	8.015 ± 0.039 (16)	8.007 ± 0.029 (7)	7.910 ± 0.023 (16)		
Pedal blood ammonia (μM)					
High salinity (31‰)	187 ± 14 (5)	203 ± 18 (18)	123 ± 16 (7)		
Low salinity (19‰)			129 ± (18)		
Branchial blood ammonia (μM), 20–33‰					
Postbranchial			34 ± 6 (11)		
Prebranchial			91 ± 10 (10)		

into their shells. Ventilation is virtually blocked and there is little evidence of locomotion. Local observers believe that the animals migrate offshore and "hibernate". While the movements of populations are poorly known, it is clear that the most active period of feeding and locomotion spans less than half of the year.

Blood osmotic and ionic composition

The blood of warm acclimated (22° C) animals tends to be slightly (but not significantly, $P > 0.05$) hyperosmotic to the medium throughout the range of salinity tolerance (Table I). The hypersomotic condition of the blood, which grows larger at low temperature, becomes highly significant at 6° C ($P < 0.001$). As shown earlier (Mangum and Lykkeboe, 1979), the blood at 22° C is consistently hypoionic to the medium with respect to NaCl and Mg^{+2}, isoionic in K^+, and hyperionic in Ca^{+2}, NH_4^+ and HCO_3^-, regardless of acclimation salinity. At low temperature, the blood-medium difference in inorganic osmolytes decreases (Mangum and Lykkeboe, 1979), which more than accounts for the changes in osmolality (Table I).

Pedal blood pH rises at low temperature, at least in the range 10 to 22° C (Table I), but the change is quite small ($\Delta pH/\Delta C = -0.008$ at both salinities). When the temperature of a blood sample held *in vitro* is changed anaerobically, the change remains smaller (-0.012) than that of pN, the neutral point of water, over the same temperature interval ($\Delta pN/\Delta C = -0.014$). No further increase in blood pH occurs *in vivo* at 6 to 10° C (Table I), which may be due to an accumulation of acidic metabolites in the absence of blood flow (see below).

Blood pH rises ($P < 0.05$) at low salinity (Table I), but this change is also much smaller than observed in other groups of euryhaline animals (Mangum, 1976).

Intracellular free amino acids and nitrogen excretion

The size of the total free amino acid pool in pedal muscle cells varies directly with acclimation salinity (Table II). However, the change is very small and the pattern differs from that in lamellibranchs, which has been investigated more intensively. Alanine drops sharply at low salinity, as in other gastropods (Schoffeneils and Gilles, 1972; Bedford, 1971) and ornithine decreases appreciably as well, neither of which could have been detected by the methods used in previous

TABLE II

The concentration (μM/g dry wt) and % composition of free amino acids in pedal muscle fibers of Busycon canaliculatum *acclimated to high and low salinity at 23 to 24° C.*

	18‰		33‰		ΔμM/g
	μM/g	% total	μM/g	% total	
taurine	114.2	46.8	94.1	31.7	−20.1
aspartic acid	26.1	10.7	28.1	9.5	2.0
threonine	7.2	3.0	3.6	1.2	−3.6
serine	13.5	5.5	11.7	3.9	−1.8
glutamic acid	24.5	10.0	18.3	6.2	−6.0
proline	19.3	7.9	17.4	5.9	−1.9
glycine	3.1	1.3	10.4	3.5	7.3
alanine	15.0	6.2	57.6	19.4	42.6
valine	—	0	7.1	2.4	7.1
methionine	3.8	1.6	—	0	−3.8
isoleucine	1.3	0.5	4.9	1.7	3.6
leucine	1.8	0.7	10.5	3.5	8.7
tyrosine	—	0	4.8	1.6	4.8
phenylalanine	2.2	0.9	2.0	0.7	−0.2
ornithine	1.7	0.7	20.1	6.8	18.4
lysine	6.8	2.8	6.1	2.1	−0.7
histidine	3.6	1.5	—	0	−3.6
Total	243.9	100.1	296.7	100.1	52.8

investigations. These changes are offset in large part by an increase in taurine, which has been reported before but not in this magnitude (Schoffeneils and Gilles, 1972). The net free amino acid change of 53 μM/g dry wt is only 17% of that in the clam *Rangia cuneata,* for example, over a comparable salinity interval (Henry and Mangum, 1979). Assuming the same intracellular water content in the two species, the molal change in *B. canaliculatum* (164 mM/kg cell H_2O) is only half that in the related neptuneid *Buccinum undatum,* over the same salinity range (Staaland, 1970). In view of the small change in free amino acids, it is not surprising that the levels of ammonia in the blood (Table I) do not change significantly with acclimation salinity. Nor is there a significant rise in ammonia and free amino acid excretion (Table III) at low salinity ($P < 0.05$).

The magntude of nitrogen excretion is somewhat unexpected. The total in *B. canaliculatum,* a predator and carrion feeder, is an order of magnitude lower

TABLE III

Nitrogen excretion in Busycon canaliculatum *Mean ± s.e. (N).*

Experimental (=acclimation) conditions	Ammonia excretion [μM/(g dry wt·hr)]	Free amino acid excretion [μM leucine/(gm dry wt·hr)]
A. Paired observations on intact animals		
15° C, 18‰	0.54 ± 0.05 (7)	0.33 ± 0.50 (7)
15° C, 30‰	0.49 ± 0.06 (7)	0.36 ± 0.41 (7)
B. Pedal muscle tissue		
5° C, 31‰	0.62 ± 0.03 (8)	
25° C, 31‰	1.10 ± 0.15 (8)	

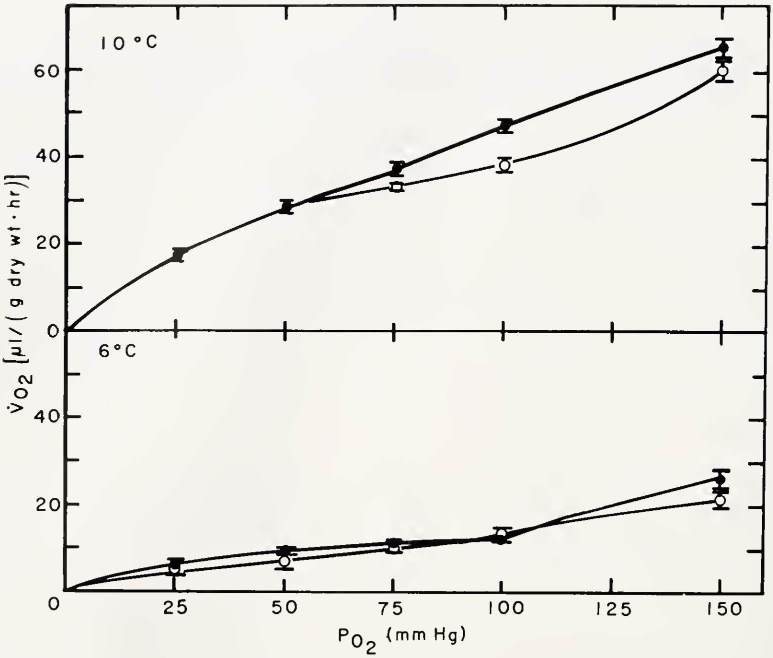

FIGURE 1. The influence of temperature and salinity on total oxygen uptake ($\dot{V}_{O_2}$) in *Busycon canaliculatum*. (Closed circles 31 to 34‰, (open circles) 18 to 19‰. Mean ± s.e. (N = 9).

at 15° C than in *R. cuneata*, an herbivorous filter feeder, at 22° C and the same acclimation salinity (18–22‰). This difference is unlikely to be due solely to the lower experimental temperature. At the slightly higher experimental temperature of 25° C, ammonia production of isolated muscle fibers taken from *B. canaliculatum* (Table III) is also less than in *R. cuneata* [3.6 µM/(g·hr) at 22° C; Henry and Mangum, 1980]. On the other hand, the magnitude of ammonia excretion in both animals and isolated muscle fibers is directly correlated with the rate of oxidative metabolism, which is considerably higher in *R. cuneata* (Henry and Mangum, 1980).

Oxygen uptake

The complex relationship between oxygen uptake and ambient P_{O_2} (Fig. 1 in Mangum and Polites, 1980) is not influenced by acclimation salinity, but the exponential change at high P_{O_2} becomes very small at low temperature (Fig. 1 in present report). Using paired observations on the same animals, the influence of a salinity change in either direction is clear. In 8 of the 9 individuals tested, oxygen uptake varies directly with acclimation salinity, and the mean decrease at low salinity, 22° C and ambient P_{O_2} 100 to 125 mm Hg is 26 (± 6) %. No paired observations were made at lower temperatures.

Oxygen uptake decreases sharply at low temperature. Using the data for both acclimation salinities, which do not differ significantly, the weight-corrected value for a 24.2-g animal at 100 to 125 mm Hg is 36 (± 3 s.e.) µl/(g dry wt·hr) (N = 28) at 10° C and 12.6 (± 2.5) µl/(g dry wt·hr) (N = 16) at 6° C. The

TABLE IV

Oxygen uptake ($\dot{V}_{O_2}$) of tissues isolated from Busycon canaliculatum. *18‰, 22° C. Mean ± s.e. (N).*

Tissue	$\dot{V}_{O_2}$ [μl/(g dry wt·hr)]	Difference from 35‰ (%)
Pedal muscle	87 ± 7 (29)	−22 ($P < 0.05$)
Cardiac muscle	484 ± 20 (5)	not significant
Pedal epidermis	235 ± 19 (5)	not significant
Gill	961 ± 26 (12)	not significant

temperature coefficients (Q_{10}) for total oxidative metabolism are 2.0 for the interval 10 to 22° C, and 14 for 6 to 10° C.

While the oxygen uptake of pedal muscle tissue taken from low salinity acclimated animals is significantly lower (Table IV) than that of high salinity-acclimated animals (Mangum and Polites, 1979), the rate in other isolated tissues does not change significantly ($P > 0.05$).

Ventilation

In warm-acclimated animals at 22° C, ventilation decreases by 17% to 13.4 ml/(g dry wt·hr) at 18‰ salinity, possibly due to a direct effect of low salt levels on the activity of the ciliary pump, as in Lamellibranchia (Ghiretti, 1966). At low temperatures, retraction into the shell results in a decrease of the cross-sectional area of the excurrent to a size that is too small for the probe to function without altering the flow properties of the stream, and the siphon will not extend far enough to make a tight connection with a manometer. Under these conditions no measurements were made.

Blood P_{O_2}, oxygen carrying capacity and oxygenation

Blood P_{O_2} does not change significantly with salinity, regardless of temperature (Table V), but it rises at 10° C. More notably, the relationship of blood P_{O_2}

TABLE V

Effect of acclimation salinity and temperature on the respiratory properties of the blood in Busycon canaliculatum. *Mean ± s.e. (N). Data for 31 to 34‰ and 22° C from Mangum and Polites (1979).*

	22° C		10° C	
	31–34‰	18‰	31–34‰	18‰
Postbranchial blood				
PO_2 (mm Hg)	25	25 ± 3 (6)	45 ± 6 (4)	48 ± 7 (3)
Hcy O_2 (%)	84	73	100	100
Prebranchial blood				
PO_2 (mm Hg)	2.3	2.1 ± 0.3 (5)	19 ± 3 (4)	19 ± 3 (5)
Hcy O_2 (%)	16	16	86	86
Pedal blood				
PO_2 (mm Hg)	5.5	7.1 ± 2.0	62 ± 8 (8)	
Hcy O_2 (%)	33	40	100	
Nephridial blood				
PO_2 (mm Hg)			12 ± 1 (3)	
Hcy O_2 (%)			82	
Postbranchial-prebranchial blood O_2 conc. (ml/100 ml)	2.25	1.89	0.78	

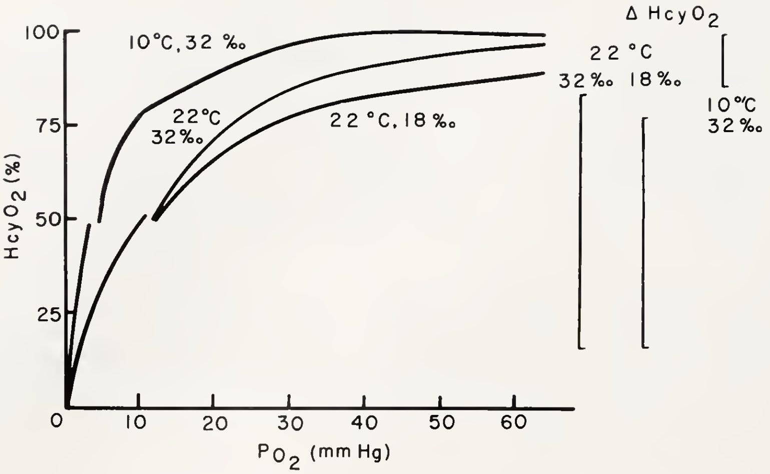

FIGURE 2. The influence of temperature and salinity on oxygen transport in the blood of *Busycon canaliculatum.* Vertical bars at right showing change in hemocyanin oxygenation. Data from Table V.

in different parts of the system shifts at low temperature. At 10° C, the margin of the partly retracted foot appears to be swollen with dark blue blood, which is more highly oxygenated than postbranchial blood (Table V). The hydrostatic pressure generated in the posture seems to distend the pedal epithelium, and it should reduce the diffusion distance between pedal blood and ambient water. Apparently the foot becomes a site of oxygen uptake.

If the volume of the blood increases at low salinity by about 15%, as in *Buccinum undatum* (Staaland; 1970), then oxygen carrying capacity should decrease measurably. In a total of 20 determinations on pedal blood and 26 on renal blood at various temperatures, there was no detectable effect of acclimation salinity. Although the copper content of blood diminishes in winter animals, presumably below 10° C (Betzer and Pilson, 1974), there is no evidence in our data of a significant decrease ($P < 0.50$) in the oxygen-carrying capacity of blood taken from animals acclimated to 10° C. In contrast to warm-acclimated animals (Mangum, 1979), there is no difference in absorbance at 345 nm between samples of pedal and renal blood ($N = 26$), suggesting little or no dilution of pedal blood, when the animals are retracted.

Using the data for oxygen equilibrium properties reported earlier (Mangum and Lykkeboe, 1979), the effect of low temperature and low salinity on the respiratory properties of the blood is summarized in Table V and Figure 2. The removal of divalent cations from the blood at 18‰ significantly reduces the cooperativity of oxygen binding to *B. canaliculatum* hemocyanin, which appreciably lowers its oxygenation state at a particular pH and P_{O_2}. The small increase in pH at low salinity does not significantly influence oxygenation, and there is no compensatory increase in P_{O_2} to enhance oxygenation. The net result is a decrease of about 16% in oxygen uptake by the blood. The oxygen uptake of many tissues is not directly influenced by acclimation salinity, but $\dot{V}_{O_2}$ of pedal muscle fibers decreases, which may account for the discrepancy between the estimated decrease of blood-supplied oxidative metabolism (16%) and the observed decrease of total oxidative metabolism (26%).

At 10° C the critical pH at which the reverse Bohr shift disappears increases to about 8.05, and thus the oxygen affinity of the blood increases more ($\Delta H = -17$ kcal/mole at pH 8.0) than it would if the Bohr shifts were not heterogeneous or if they were independent of temperature (Mangum and Lykkeboe, 1979). While the blood is more highly oxygenated at the gill at 10 than at 22° C, the venous reserve is also larger (Table V). Oxygen extraction from the blood decreases from 82% at 22° C to only 43% at 10° C. Using the erroneous assumption that oxygen uptake by the blood equals total oxygen uptake, the Fick estimate of cardiac output predicts an increase of 96 ml/(kg·min). A single successful measurement of heart rate, however, showed a substantial decrease (> 50%), which is unlikely to be reversed by a larger stroke volume. The discrepancy between the changes in heart rate and the Fick estimate of cardiac output suggest an increase in the relative importance of direct oxygen uptake by unvascularized tissue, which exaggerates the error in the Fick estimate. This suggestion is strongly supported by the data for blood P_{O_2}, which are distributed in the system differently at 10° C, and also by the change in total oxygen uptake (−56%), which agrees with that of heart rate.

At 6° C, we were unable to detect a heartbeat by visual observation of a large number of individuals, or by implanted electrodes (de Fur and Mangum, 1979). Although no measurements were attempted on the blood taken from sites such as the gill, now withdrawn deep into the shell, pedal blood P_{O_2} remained very high [70 (± 12 s.e.) mm Hg, $N = 6$].

Discussion

Blood pH in *Busycon canaliculatum* is relatively insensitive to environmental change, which precludes the possibility of restoring its respiratory function by opposing thermal or ionic effects with a concomitant alteration of acid-base status. Although the insensitivity is in part an intrinsic property of the blood, it is not easily explained by the ionization of structural groups on the hemocyanin molecule. If gross histidine content were the only factor determining the buffer properties of a protein-containing blood, the thermal behavior of conch blood would be the same as that of mammalian blood; both prosobranch hemocyanin and Hemoglobin A contain about 6% histidine (Van Holde and Van Bruggen, 1971). The departure cannot be attributed to the absence in conchs of other imidazole-containing proteins, which does not result in reduced thermal sensitivity of pH in many groups of animals (Mangum, 1973). In its thermal behavior *B. canaliculatum* blood, essentially a mixture of sea water and hemocyanin, resembles sea water ($\Delta pH/\Delta C$ of the CO_2 system $= -0.007$) more than a high imidazole-containing solution ($\Delta pH/\Delta C = -0.018$; Reeves, 1972). We suggest that the basis of the similarity is the unique character of *Busycon* blood, which *is* more sea water than protein in the quite literal sense (Mangum, 1979). At summer temperature the foot is apparently inflated with sea water, which appears to mix freely with blood, and it is blood pH at these temperatures which is unusual, not the values at 6 to 10° C. The direction of this departure, or an unusually high pH at 23° C, is the expected trend if the CO_2 system of sea water dominates the heterogeneous buffer system in the blood.

The relatively small increase in blood pH at low salinity may be due in large part to the very small change in intracellular free amino acids and concomitant alkalinization by ammonia. A similar correlation, a small change in blood pH with acclimation salinity in an animal that does not utilize free amino acids as intracellular osmolytes, is found in the xiphosuran *Limulus polyphemus* (Mangum, Booth, deFur, Heckel, Henry, Oglesby and Polites, 1976). Unlike *Limulus*, the excretion of hydrogen ions *via* ammonia is very small in *Busycon*.

Alternatively, or perhaps in conjunction, it has been suggested that the component of the CO_2 system which is regulated homeostatically in decapod crustaceans is not H^+ but HCO_3^- (Truchot, 1978), which is believed to be actively excreted in exchange for Cl^-. Unlike these species, in which acd-base regulation has been studied in detail, *B. canaliculatum* is capable of only the most trivial ion regulation, and by no means is it clear that the process involves an active ion exchange. The machinery to maintain constant bicarbonate levels may simply be lacking in conchs. Our data on CO_2 levels in the blood are far too few to permit a meaningful conclusion, and it is clear that the subject of acid-base balance in osmoconformers is in need of serious investigation.

Regardless of its explanation, the conservatism of blood pH in conchs is not mysterious from an adaptive point of view. If the thermal response were determined by an imidazole alphastat, prebranchial blood pH at 23° C would be 7.7 and postbranchial pH 7.8, and the reverse Bohr shift would be a genuine physiological anomaly. The blood would return to the gill with fully 30% of its oxygen load, oxygenation at the gill would increase only to 89%, and oxygen uptake into the blood would be 12% less than it is. Similarly, a pH increase of 0.15 to 0.20 at low salinity, as in crabs, would not affect oxygen affinity, because it would occur in the pH range where the Bohr shift is virtually nonexistent (> 7.9). But a hypothetical increase in pH would elevate the carbonate content of the blood, enhancing the formation of Ca^{+2} and Mg^{+2} ion-pairs; cooperativity would probably disappear entirely and oxygen uptake at the gill would be further impaired. In fact, oxygen uptake is reduced at both low temperature and low salinity, and the change clearly results in part from unopposed effects of the environmental variables on the blood oxygen carrier. We suggest that these effects are important in limiting the ecological range of the species, and in determining its behavior.

Our findings on the oxygenation state of postbranchial blood, and on the high mortality rate in the laboratory, suggest that 22 to 24° C is slightly above the optimum, which is supported by popular accounts of "estivation" or the disappearance of conchs in the heat of mid-summer. At a temperature only a few degrees lower (19° C), little mortality occurs in either running or recirculating water and a few observations suggest that total oxygen uptake may even increase possibly due to complete oxygenation of the carrier at the gill.

The oxygen transport system appears to be specilized for transitional and early summer temperatures. Below 10° C, corresponding to the period of about 6 months when conchs terminate active feeding and locomotion, the increase in oxygen affinity (to less than 2 mm Hg at physiological pH) effectively precludes the possibility of drawing on the oxygen supply in the blood. The temperature dependence of $\dot{V}_{O_2}$, as described by Q_{10}, abruptly rises. It is hardly surprising that the copper concentration of the blood falls, implying the suspension of hemocyanin synthesis during this period (Betzer and Pilson, 1974), which results in a drop in oxygen carrying capacity (calculated from Betzer and Pilson, 1974) to one-fourth of the peak value in the middle of the summer. Given the intrinsic physico-chemical sensitivity of conch hemocyanin and the limitations of the fluid convection systems, it is difficult to conceive of a compensatory physiological response that could offset the effects of the greatly increased oxygen affinity. We suggest that more active blood or water convection might entail little or no net energetic advantage, and that the suspension of activity for half of the year is an adaptive response necessitated by the thermal sensitivity of the respiratory and metabolic systems.

The ecological limits of the oxygen transport system of *B. canaliculatum* are even more obvious at low salinity. As concluded earlier (Mangum and Lykkeboe, 1979), the intrinsic sensitivity of hemocyanin oxygen affinity to the loss of blood NaCl accompanying the invasion of estuarine waters appears to be offset by complex interactions of the molecule with the divalent cations and the anions of the CO_2 buffer system, resulting in little or no change. The response of P_{50}, however, is of little physiological importance in this species, in which the relevant oxygenation states are P_{85}, P_{15}, etc., or those at physiological P_{O_2} (see Table V). Above 40% these oxygenation states are strongly influenced by cooperative interactions of the oxygen-binding sites, which in turn depend on the divalent cations in the blood. Since the relationship is exponential in the physiological range, the small changes in divalent cations accompanying acclimation to low salinity can have large effects on aerobic respiration. If cooperativity were eliminated entirely while oxygen affinity remained unchanged, a very real possibility of further penetration into the estuary, oxygenation at postbranchial blood P_{O_2} would be only 64%, net oxygen uptake into the blood would drop to 1.60 ml/100 ml and the oxygen supply to the tissues would be half that observed (Table V, Fig. 2). Since the species does not actively regulate the osmotic concentration of its blood and since the mechanisms of reducing cell osmolytes is largely unknown, it is not obvious that the energetic demands of living in low and high salinity waters are very different. There is no reason to suppose, however, that the oxygen requirements compatible with life go down at low salinity. Although there are undoubtedly others, the determinants of salinity tolerance may include the properties of the oxygen transport system, which simply does not work as well in dilute waters.

SUMMARY

1. The oxygen transport function of the blood in *Busycon canaliculatum* diminishes when either the ambient temperature or the ambient salinity is lowered.

2. At low temperature the oxygen affinity of the hemocyanin increases sharply, and blood P_{O_2} increases as well. Thus the blood cannot deoxygenate at the tissues.

3. At low salinity oxygen affinity does not change appreciably, but the loss of Ca^{+2} and Mg^{+2} from the blood reduces the cooperativity of hemocyanin-oxygen binding, requiring a higher P_{O_2} to achieve a given oxygenation state. Thus the blood does not oxygenate as highly at the gill, and total oxygen uptake decreases.

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LIFE CYCLE OF LABORATORY-REARED TOBACCO HORNWORMS, *MANDUCA SEXTA*, A STUDY OF DEVELOPMENT AND BEHAVIOR, USING TIME-LAPSE CINEMATOGRAPHY

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The tobacco hornworm, *Manduca sexta* (L.), is a multivoltine leaf feeder that grows rapidly up to 10 g in the larval stage and has been studied since the start of the tobacco industry. The development of controlled laboratory rearing techniques (Yamamoto, 1969; Bell and Joachim, 1976) and its large size have promoted the use of this insect as a general lepidopteran representative for many biological studies. There is now an extensive accumulation of knowledge about *M. sexta* from field studies and from physiology and endocrine studies (Madden and Chamberlin, 1945; Peterson, 1912; Reinecke, Gerst, O'Gara, and Adams, 1978; Truman, 1972; Borg and Marks, 1973; Bell, Rasul, and Joachim, 1975; Bollenbacher, Vedeckis, Gilbert, and O'Connor, 1975; Sandburg, Kramer, Kezdy, and Law, 1975; and Kramer, Dunn, Peterson, Seballos, Sandburg, and Law, 1976). However, a complete description of the developmental and behavioral events of this insect in the laboratory has never been reported. Such information is extremely important in the rearing and use of this insect for research and when more precise staging of physiological age is needed. Accurate staging is critical in endocrine studies of lower animals such as insects because hormone releases are usually of very short duration and frequently induce a rapid change in behavior (Truman and Riddiford, 1977; Truman and Endo, 1974).

This paper reports the application of time-lapse cinematography for observations on growth, development, and behavior of laboratory-reared specimens of *M. sexta* to determine more accurate timing of morphological, physiological, and behavioral events.

MATERIALS AND METHODS

Rearing procedures

Specimens of *M. sexta* were reared by the method of Bell and Joachim (1976), but the diet used was a high wheat germ type developed for mass rearing the gypsy moth, *Lymantria dispar* (L.) (Bell, Owens, Shapiro and Tardif, 1980). This diet was altered to contain 0.24% formaldehyde and 10% less water (to increase the firmness of the congealed diet). Rearing temperature was maintained at 25° ± 0.2° C, and the relative humidity was held between 20 and 40%. A diapause-inducing light regimen was used (12L: 12D). Lights-off was at 6:00 P.M., which was arbitrarily designated as 2400 (00:00) A.Z.T. (Truman, 1972).

Preparation of containers for photography

Standard rearing cups (Bell and Joachim, 1976) were used for multiple photographic recordings of up to 11 insects. The 30-ml cups were placed on their sides, and the 120-ml cups were placed upside down, which allowed photographic observations at low magnifications.

For more detailed observations at higher magnification, two sizes of containers were constructed from clear plexiglass tubing with 3.2 and 7.0 cm inside diameters. They were 5 and 8 cm high, respectively. Both were covered with glass lids and placed on a plexiglass sheet that served as the container bottoms and the photographic platform. The bottom of the 3.2-cm container was covered with a 5-mm thick layer of diet, which was sufficient to maintain the larvae through the fourth instar. The larger container was used for fourth-instar insects through pupation and blocks of diet were supplied every two days (at which time the frass was removed) until feeding stopped. At the cessation of feeding, the bottoms of the containers were occasionally lined with tough brown absorbent paper.

A third photographic setup was constructed from two 70 × 50-cm glass plates that were separated by 2 cm with a wooden frame. Three-fourths of this container was filled with soil and placed on the long edge for time-lapse photography of nondiapausing fifth-instar insects from cessation of feeding through pupation and again at 18 days to record eclosion.

During filming, an electric wrist watch with date, a small centigrade thermometer, a 20-mm ruler, and a data card were placed in the field of view of the cylindrical containers. A clock was used with the soil-filled container.

Procedures for selection of insects

Except where noted, only the physiologically advanced (PA) specimens of *M. sexta* were used. The PA insects represented about 10% of the total population at the time of this study and were chosen because of their greater synchrony and uniformity as a group and the ease in which they could be selected.

When an entire larval cycle was recorded by time-lapse cinematography, there was a slight retardation. This was avoided by recording only a part of the life cycle at any one time and comparing it with the progress of the insects remaining within the standard colony. Thus, timing records were compiled from a number of filmed sequences with durations of 6 to 12 days.

Eggs for the more critical time studies were collected from a tobacco plant that had been in the egging chamber between 11:30 PM and 12:30 AM. Insects hatching from these eggs were photographed through the second ecdysis only. Records were made for only the most rapid developers.

Photographic equipment

An Arriflex 16-mm movie camera adapted for time-lapse and flash synchronization was used for all filmed studies. The photographic illuminator was a Chadwick-Helmuth lamp (model 71) powered by their Strobex 136–M1. The electronic flash was used without the accompanying condenser lens, and a flash meter (Calumet M-100) was used to measure the light output for film exposure (the film was Eastman 7240, 16-mm ASA 80, reversal color with 85B filter). A filming rate of one exposure per five min was commonly used for the time-lapse recordings. The film was viewed with an L.W International data anlyzer projector (224 AMK IV).

Environmental conditions for photography

Filming of the animals was conducted in a controlled environmental room at the same conditions as described for rearing. Since diapause in *M. sexta* is inhibited by a light interruption applied shortly after the onset of the dark period (Bell, Rasul, and Joachim, 1975), effects of the electronic flash on diapause was examined by placing the electronic flash 60 cm from a group of 20 newly ecdysed fifth-instar larvae. The flash was triggered every 30 sec at maximum intensity (about 75 joules/flash with a flash duration reported to be 135 microseconds) for the duration of the instar. All insects entered diapause, and no other effect of the flash was noted.

Analytical procedures

The date and time of day were recorded from the films for each event. Time from oviposition (chosen as 12:05 AM on the night oviposited) to each event was determined, and means and standard deviations were calculated for the onset of events and, again, for the duration of events. The data from obviously aberrant insects were discarded. The percentage of time elapsed to pupation and A.Z.T. times were derived from the data on time to occurrence. Data on average weight were obtained from standard colony insects (not just PAs) that were selected during known weight-stable stages.

TABLE I

Time of onset of events in days, percentages, and A.Z.T.[*] *time for physiologically advanced (PA)*[†] *specimens of* M. sexta.

Event	Time at onset of events in days	N	Percentage of development at onset of events	A.Z.T. time of day in hr
Oviposition	00.00 ± 0.01[‡]	11	00.00 ± 0.05[‡]	06.0 ± 0.3[‡]
First-instar hatch (L1)	4.03 ± 0.02	4	17.71 ± 0.11	06.8 ± 0.6
Molt sleep	5.78 ± 0.02	4	25.38 ± 0.09	00.7 ± 0.5
L1-L2 ecdysis	6.37 ± 0.02	11	27.96 ± 0.08	14.8 ± 0.4
Molt sleep	7.82 ± 0.06	10	34.36 ± 0.27	01.8 ± 1.5
L2-L3 ecdysis	8.41 ± 0.06	10	36.92 ± 0.26	15.8 ± 1.4
Molt sleep	9.97 ± 0.10	8	43.79 ± 0.41	05.3 ± 2.3
L3-L4 ecdysis	10.65 ± 0.10	8	46.75 ± 0.42	21.5 ± 2.3
Molt sleep	12.68 ± 0.09	7	55.69 ± 0.41	22.3 ± 2.2
L4-L5 ecdysis	13.71 ± 0.11	11	60.21 ± 0.50	23.0 ± 2.7
Coated fecal pellets	15.76 ± 0.04	4	69.21 ± 0.18	00.2 ± 1.0
Cessation of feeding	17.61 ± 0.15	11	77.32 ± 0.69	20.5 ± 3.7
Heart exposure	17.72 ± 0.12	5	77.83 ± 0.53	23.4 ± 2.9
Body wetting	17.81 ± 0.08	11	78.20 ± 0.36	01.4 ± 1.9
Wandering	17.92 ± 0.08	11	00.00 ± 0.05	06.0 ± 0.3
Dorsal pigmentation	17.98 ± 0.20	3	78.94 ± 0.88	05.4 ± 4.8
Burrowing	18.27 ± 0.19	11	80.25 ± 0.85	12.6 ± 4.6
Fluid excretion	18.61 ± 0.22	7	81.73 ± 0.98	20.6 ± 5.4
Reduced movement	19.23 ± 0.20	11	82.59 ± 0.88	01.3 ± 4.8
Stationary stage	19.75 ± 0.10	11	86.74 ± 0.42	00.0 ± 2.3
Metathoracic bars	22.11 ± 0.20	11	97.11 ± 0.87	08.7 ± 4.8
Larval-pupal ecdysis	22.77 ± 0.20	11	100.00 ± 0.88	00.5 ± 4.8

[*] A.Z.T. time is 0 at onset of 12-hr scotophase, which was 6:00 PM in this study. Thus heart exposure occurred 23.4 hr after onset of scotophase and took place in photophase, of which there were 0.6 hr left.

[†] PA = insects selected as most physiologically advanced (about 8 or 9% faster than standard colony), held at 25° C and 12-hr photophase.

[‡] Data represent the mean and s.d. for the numbers of insects (N) as indicated.

TABLE II

Duration of events of PA-selected specimens of* M. sexta *and weight of non-PA selected insects during weight-stable stages.*

Event	Mean duration of events in hr	N	Mean weight	N
Egg stage	96.8 ± 0.6**	8	1.41 ± 0.10 mg**	20
First-instar feeding	42.0 ± 0.7	4	1.03 ± 0.07 mg†	11
Molt sleep	14.1 ± 0.5	4	7.64 ± 1.29 mg	20
Second-instar feeding	35.0 ± 1.9	11		
Molt sleep	14.0 ± 1.6	11	30.40 ± 2.70 mg	8
Third-instar feeding	37.5 ± 3.7	11		
Molt sleep	16.2 ± 0.8	6	0.20 ± 0.02 g	21
Fourth-instar feeding	46.6 ± 0.9	7		
Molt sleep	24.7 ± 2.0	8	1.08 ± 0.09 g	20
Fifth-instar feeding	90.6 ± 3.8	11		
Cessation of feeding to body wetting	4.8 ± 1.6	11	9.23 ± 0.53 g	10
Body wetting	2.7 ± 0.7	11		
Wandering to burrowing	8.5 ± 2.9	11		
Dorsal pigmentation††	27.0 ± 8.4	3		
Fluid excretion††	28.3 ± 7.4	6		
Burrowing to reduced movement	22.9 ± 5.5	11		
Reduced movement	12.9 ± 3.9	9		
Stationary stage to pupation	72.5 ± 4.3	11		
Metathoracic bars to pupation††	15.8 ± 1.4	11	5.02 ± 0.63 g	10
Pupation to eclosion—diapause	2,328 ± 720‡	567	4.71 ± 0.62 g	30
Pupation to eclosion—nondiapause	523 ± 31‡	26		

* Insects selected as most physiologically advanced (~10% of colony population), held at 25° C and 12-hr photophase.

** Data represent the mean and s.d. for the number of insects (N) as indicated.

† Weight taken immediately after hatch.

†† Overlapping events.

‡ Duration of diapause (obtained from one-month data of standard colony) = 97 ± 30 days. Duration of nondiapause (induced with 15 hr light: 9 hr dark) = 21.8 ± 1.3 days.

RESULTS

Data obtained from filming the growth and development of individual laboratory-reared specimens of *M. sexta* were summarized, and the timing for major physiological and behavioral events is presented in tabular form. Table I lists the events monitored in order of occurrence, the mean number of days per event, and the mean percentage of time elapsed btween events from oviposition to pupation (100%). Table II shows the mean duration of most events in hours in order of occurrence, with the mean weights of insects selected from the standard colony during known weight-stable stages.

Oviposition

A time-lapse recording was made of adult insects in the egging cage in an effort to determine whether there were any general activity cycles. Flying adults were momentarily affected by each electron flash (5-min intervals), but egg harvest was normal. It was found that oviposition, periods of rest, feeding, and mating occurred randomly under simulated moonlight conditions (dimmed tungsten bulb emitting 3 lux at 20 cm) throughout the 12-hr dark period. Activities started and stopped about five min after the onset of the dark and light cycles, respectively, and there was no effective activity during the light period. Thus, oviposition occurred between 6:05 PM and 6:05 AM, and the average time of oviposition for all specimens was assumed to be 12:05 AM.

Larval growth and development

Egg hatch and larval ecdyses. Larval emergence from the egg occurred 4 days after oviposition (Table I), and the mean weight immediately after hatch was about 1.4 mg (Table II). The first-instar larvae had silk glands that produced a strand on which they could descend. Also, a small mass of silk was frequently found tangled around the prolegs at the end of this instar. Ecdysis took less than 1 min to complete for all larval stages, as observed by eye. Between the onset times of larval ecdyses (Table I), the average length of time spent in the first through fourth instars was 2.3, 2.0, 2.2, and 3.1 days, respectively.

Feeding. Immediately after egg hatch, most larvae consumed their egg cases before migrating a short distance to feed on diet. During the first instar, larvae fed for a few minutes, withdrew their heads from the food, and then either remained motionless or moved the head and thorax regions back and forth. The first-instar feeding stage lasted about 42 hr from eclosion, and during this time, the insects showed a 7.4-fold increase in weight (Table II). Second, third, and fourth instar larvae frequently ingested part of their cast skin soon after ecdysis and before feeding on diet. The feeding stages from eclosion for these three instars lasted 35, 38, and 47 hr, respectively (Table II). Film records of newly eclosed fourth and fifth instars showed a feeding delay of 2.3 ± 1.0 hr ($n = 8$) and 2.9 ± 2.0 hr ($n = 10$), respectively. Similar delays in feeding were assumed for the first through third instars. Feeding behavior during the fifth instar was studied in detail; a single fifth-instar larva was studied over a 45-hr period, and durations of feeding and nonfeeding were highly variable. For this larva, mean duration of a feeding period was 15 ± 20 min ($n = 71$ consecutive feedings), and duration of a nonfeeding period was 20 ± 20 min ($n = 70$). The majority of feeding contacts was of short duration, lasting about 5 min, and the longest was 150 min. Another fifth-instar larva fed at mean intervals of 41 ± 56 min ($n =$ 21 consecutive feedings), and had mean nonfeeding intervals of 19 ± 13 min ($n =$ 21 intervals) over a 24-hr period.

The feeding stage of all instars was easily interrupted by external disturbances such as the approach of an observer. Such interruptions were most noticeable for the larger fifth-instar larvae. When startled, the larvae remained motionless for several minutes; thus, frequent disturbances could possibly affect growth rate. To determine whether the photographic procedure was seriously affecting feeding behavior, fourth- and fifth-instar larvae, (shortly after ecdysis) were allowed to feed under standard rearing conditions for 40 hr. Then the number of fecal pellets was counted. In all instances, the number of pellets was similar to those determined by film analysis. Thus, the photographic apparatus did not interfere with feeding. The highest rate of pellet production observed by film analysis was one every 19 ± 3 min ($n = 41$) for fifth-instar larvae the third day after ecdysis Pellet production was not critically determined for the first three instars.

Rate of growth and weight-doubling time. Fifth-instar larvae gained 90% of their weight (9.23 g) during a feeding period of 91 hr (Table II). However, this instar had the slowest rate of growth, as shown by a weight-doubling time of 21.2 hr (Table III). A weight-doubling time of 17.6 hr for second-instar larvae was more than 1.5 times as long as that determined for first- and third-instar larvae although it was the same as for the fourth instar. Figure 1 depicts a graphic

TABLE III

Feeding time per instar needed for M. sexta *larva to double in weight.*

Instar	Weight-doubling time (hr)*
First	11.3
Second	17.6
Third	11.4
Fourth	17.3
Fifth	21.2

* Duration of each feeding stage derived from Table II as well as weight at hatch, during molt sleep, and at cessation of feeding of fifth-instar larvae.

perspective between growth rate and life cycle activities through pupation. The rapid growth rate for first and third instar larvae is easily seen by the slope of the plot of log of percentage weight vs. percentage of time (solid line, Fig. 1). The massive weight gain of fifth-instar larvae is clearly illustrated when viewed as a linear scale and compared with the other instars (dashed line, Fig. 1).

Molt sleep. At 5.8 days after oviposition, the first-instar larvae entered a quiescent period—the molt sleep. The onset of this event occurred within a period of 10 min and was followed by a slight body pulsation that lasted about 15 min. The pulsation was associated with the displacement of the old head capsule. Similar molt sleep behavior was observed for all but fifth-instar larvae. Duration of this event was 14 to 16 hr for the first through third instars and increased to about 25 hr during the fourth instar (Table II).

Truman (1972) observed gating in the initiation of the molt to the next instar, where about half the second-instar insects initiated the molt to the third instar during the first night after the second-instar ecdysis, but the remaining insects did not until the second night. In the present study, gating was not observed in the PA insects, but the phenomenon was routinely observed in the standard colony.

Coated fecal pellets. Midway through the feeding stage, fifth-instar larvae produced fecal pellets that were coated with a chalky material identified as uric acid (Buckner, Caldwell, and Reinecke, 1980). Nijhout and Williams (1974) were the first to report this phenomenon, which they termed "frosted frass," and they observed it after 3 days of feeding into the fifth instar. The high wheat germ diet used in this study did not produce markedly coated pellets. To increase the contrast of the coating, 2 mg/ml of powdered charcoal was added to some fresh diet. The larvae were transferred to the resulting black diet 1 day after the fourth to fifth ecdysis. The insects were filmed from ecdysis to beyond the feeding stage. The film showed two periods of coating: the first was compartively light and occurred at 2.08 ± 0.04 ($n = 4$) days after ecdysis. The second and heavier coating occurred at 2.44 ± 0.10 ($n = 4$) days post ecdysis. The onset of these two events was synchronous within the group photographed, and the first coated frass coincided with the onset of scotophase. Coated fecal pellets were produced throughout the remainder of the feeding period, but there was variability of coating between pellets that ranged from heavily coated to uncoated.

Development from post feeding through pupation

Cessation of feeding. Near the end of the fifth-instar feeding stage, the rate of defecation decreased. Feeding activity abruptly stopped about 94 hr after

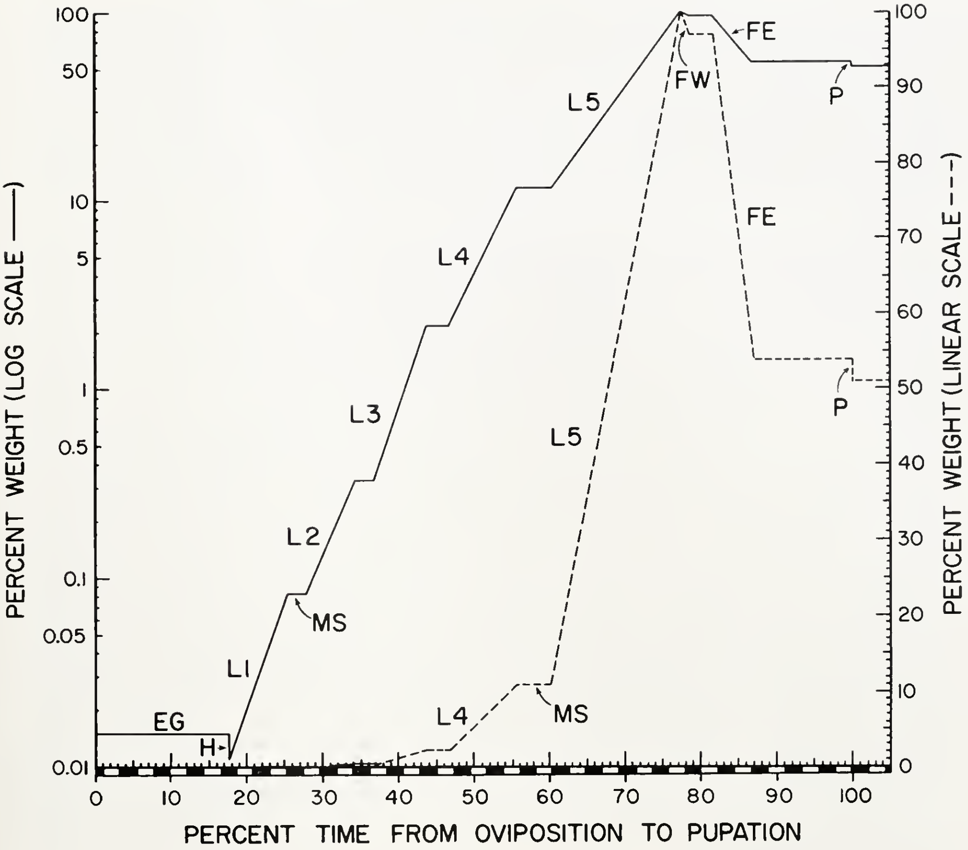

FIGURE 1. Rate of growth of physiologically advanced (PA) insects graphed on log (solid line) and linear (dotted line) scales; % weight scale (100% = 9.23 g) against % time (100% = pupation at 22.8 days). Dashes at base of graph represent 12-hr scotophases of each day at 25° C, starting at 6:00 PM. Numbers preceded by L identify instar. EG = egg stage: FE = fluid excretion ;FW = period of estimated weight loss due to end of frass elimination and use of fluid for body wetting; H = hatch; MS = molt sleep plateaus between end of feeding stage and larval-larval ecdysis; P = pupal ecdysis. All points were derived from percentage column of Table I and weight column of Table II and simply connected.

ecdysis, and then the larvae remained motionless for up to about five hours or until the onset of the scotophase.

Heart exposure. The dorsal aorta became visible approximately three hours after feeding stopped. The aorta became very obvious by the end of the next event—body wetting—and it remained so until pupal ecdysis. This visually observable event has been used frequently and is valuable as the first prodromal sign of pupation for larval *M. sexta* (Truman and Riddiford, 1974; Nijhout, 1976). However, its occurrence and detection can vary by more than 5 hours.

Body wetting. Within about 8 hours after feeding stopped, the larvae began "body wetting." This phenomenon (but not the term) was first reported by Baumhover, Cantelo, Hobgod, Knott, and Lam (1977). Time-lapse studies showed that this activity is characterized by a sharp turning of the head so that all accessible dorsal and lateral body surfaces can be covered with an oral secretion.

The insects initiated this wetting at the thorax and proceeded to cover the surface of the body to the end of the abdomen on one side and then repeated it on the other side, taking from 5 to 15 min to complete each side; the sides were alternately moistened three or four times. Mean duration of body wetting was 2.7 hr, and it tended to begin about 7:00 PM. Defecation continued throughout this stage and resulted in the production of about six fecal pellets after cessation of feeding.

Insects that were physiologically ready for body wetting started this activity at the onset of lights off. The remaining insects entered an increased state of activity until they, too, were capable of body wetting (as much as several hours later). This was the only instance we noted in which a change in lighting altered behavior. This effect was subsequently used to derive a new and better synchronized 0 time, which started with body wetting at 0 A.Z.T. A more precise synchronization of post feeding of *M. sexta* was obtained by simply selecting larvae 4 days after ecdysis and shortly before lights off and which showed the first signs of heart exposure but did not display a wet body. These insects were again observed 1 hr or so after the onset of the scotophase. Those showing body wetting characteristics (e.g., wet body or sharp turning) were selected as physiologically synchronized to within 1 hr of each other. Film records of six larvae showing the onset of body wetting within 15 min of lights off ended body wetting activtiy 3.25 ± 0.18 hr later, and all pupated 5.10 ± 0.02 days from the new time 0.

Wandering. Larvae began the wandering stage (Truman and Riddiford, 1974) immediately after body wetting activity ceased. Wandering activity was continuous, and the duration depended upon the condition of the container. When the cup was cleared of diet and frass soon after body wetting, wandering activity lasted about 32 hr. However, when residue was left in the container, duration of wandering was reduced to 8.5 hr (Table II). When the larvae were allowed to end body wetting on soil, wandering was occasionally eliminated and replaced by burrowing behavior.

Dorsal pigmentation. A reddish pigmentation spread slowly over the dorsal surface of the larvae at the end of body wetting or shortly after the onset of wandering for about 12 hr and then diminished at about the same rate. Coloration was most apparent a few hours after the onset of wandering (or burrowing). However, some larvae had no noticeable pigmentation.

Burrowing. As noted, when residue was left in the containers, the larvae changed their behavior from wandering to burrowing, the onset of which was about 11 hr after the start of body wetting; burrowing activity lasted for 23 hr. Toward the last half of this stage, the larvae formed a pupation chamber from the mixture of frass and food. When they were placed in the narrow soil chamber, film records showed that the larvae burrowed just under the surface for several hours before pursuing a zigzag course to a depth of 6 to 20 cm, after which they formed a horizontal earthen cocoon.

Fluid excretion. The fluid excretion phase (termed gut purge by Nijhout and Williams, 1974), like heart exposure and dorsal pigmentation, overlapped major events. The larvae excreted a clear, viscous fluid from the anus in several discrete discharges over a period of 28 hr. Such discharges started about 20 hr after the onset of body wetting and occurred every 5.4 ± 5.3 hr (n = three insects with a total of 20 discharges) and were responsible for the loss of nearly 40% of larval weight. Observations reported here were made while the insects were confined in containers lined with brown paper. Larvae burrowing in the soil used this discharge to dampen the surrounding soil as they packed the mixture to form an earthen cocoon.

Reduced movement. Approximately 34 hr after the onset of body wetting, the larvae immediately entered a period of reduced locomotor activity that lasted for 12.9 hr. Larvae held in containers lined with damp paper frequently scraped the paper with their mandibles. This activity probably represented the last stage in the preparation of the earthen cocoon.

Stationary stage. Reduced locomotion was followed by a stage where the insects were unable to move forward or backward. This stage began about 47 hr after the onset of body wetting and was signaled by a characteristic movement of the head and tail regions in opposite directions so as to form a Z or S shape. Alterations in body positions, of which there were 58 ± 5 (n = 9 specimens) occurred every 1.5 to 2 hr through most of this stage but increased in frequency as the insects approached pupation. Duration of the stationary stage to pupation was 2.0 days (Table II).

Metathoracic bars. At 4.2 days after body wetting or 16 hr before pupation, two areas on the metathorax, one on each side of the heart perpendicular to the axis of the larvae, began to melanize. After 6 hr, these areas were prominently melanized marks.

Larval-pupal ecdysis. Larval-pupal ecdysis occurred about 5 days after the onset of body wetting (Table I). Three postpupation observations were made, starting with proboscis growth. Formation of the proboscis, which was attached at the tip of the mid-thorax, was aided by being stretched from the head as the insect straightened from a head down posture to full length. The proboscis was fully formed 1.8 ± 0.5 hr (n = 9) after ecdysis. Melanization from the proximal end was half completed 2.6 ± 0.5 hr (n = 9) after ecdysis and complete melanization of the proboscis occurred 4.1 ± 0.6 hr (n = 8) after ecdysis. Riddiford and Ajami (1973) published a detailed description of the characterization of *M. sexta* during the first 24 hr after larval-pupal ecdysis at 26° C. Their times of occurrence for formation, and half and complete melanization of the proboscis, were reported at 1.25, 1.50, and 3 to 3.50 hr after ecdysis, respectively.

Pupal-adult ecdysis. Diapause-entrained adults eclosed an average of 97 days after pupation. However, this varied and depended upon environmental conditions and genetic makeup of the colony. Nondiapause insects eclosed about 22 days after larval-pupal ecdysis (Table II).

A time-lapse study of an insect eclosing within an earthern cocoon 20 cm below the surface of the soil showed an adult that freed itself from the pupal skin in less than 30 sec and immediately discharged about one ml of meconium. The relase of this liquid waste softened the earthen cocoon and facilitated escape. Young moths were capable of producing another meconium discharge at any time up to the first flight. The adult made a vertical ascent to the surface in about 10 min under existing conditions.

Film records of unrestricted eclosion in a 15-cm transparent cube with a diagonal 25 cm stick showed newly emerged moths had found a wing-spreading site on the stick 11 ± 7 min (n = 8) after eclosion. The moths immediately began a dorso-lateral revolution of the wings and showed the first slight signs of wing inflation. The rotation ended in about 2 min, with the wings held in a "butterfly" position. This was evenutally followed by accelerated wing inflation activity, which was completed 33 ± 11 min (n = 8) after eclosion. At 1.2 ± 0.1 hr (n = 7) after eclosion, the moths brought the wings to the characteristic "tent" position. Adults tended to use their wings 3.5 ± 0.2 hr ($n = 6$) after eclosion. The second discharge of meconium was usually released moments before or at this time. Timed events reported here with standard deviations corresponded well to similar events reported by Truman and Endo (1974).

DISCUSSION

There are a number of factors that can affect the growth and development rate of an insect colony. Such factors include temperature, humidity, photophase, basic diet, diet preparation, source of dietary ingredients, antimicrobial agents, genetic strains, size of insect containers, competition for living or feeding space, and noise. Other factors presently too subtle to understand also appear to affect growth rate of laboratory-reared insects. For example, Nijhout and Williams (1974) attributed some variability in the *M. sexta* colony to the time of year. To help obtain a more precise study of the growth and development of a *M. sexta* colony, we employed methods that reduced commonly encountered variables and misinterpretations. Such methods included the use of stable nutrition, time-lapse cinematography, filmed recordings of mutiple insects, close monitoring of time and temperature, and the selection of a specific group of insects within the colony—the most physiologically advanced group.

Data from the percentage column of Table I may be used to derive a new growth rate at a temperature other than 25° C. This can be accomplished by carefully determining onset time of one or two events such as pupation or body wetting for PA insects reared under a different constant temperature. Such preliminary temperature studies augmented by time-lapse films have allowed the development of the equation: $Y = N[0.0061 + 0.0980\ (25 - T) + 0.0085\ (25 - T)^2]$, where N represents any time of onset in Table I, and T is the new temperature in centigrade. This formula can be used to estimate the onset times of events in insects reared at different temperatures. (Gauss-Newton method used to derive the three parameters of the equation from seven events observed at 22°, 25°, and 28° C).

Thus, the results of this study should be directly applicable in most instances to other colonies of *M. sexta* to increase the awareness of behavior and the synchrony of the life cycle for better use in research.

We thank Professor C. M. Williams, Harvard University, for encouraging us to undertake this study, which has proved most useful to us; Charles K. Graham, Mathematical Statistician, of this laboratory, for his aid in interpreting some of the data, and to Gwen Peterson and Frank Joachim for colony maintenance and precise recording of data.

SUMMARY

1. Time-lapse cinematography was used with laboratory-reared specimens of *M. sexta* to obtain information on and timing of behavioral activities.

2. Insects used in this study were selected as the most advanced group within any instar and thus were the first of the gate-I insects. Use of these insects avoided timing irregularties involving the gating phenomenon. These advance insects varied less than ±0.9% s.d. from hatch through pupation.

3. A detailed description was given for each of more than 20 timed events. Emphasis was placed on the events that occurred between the fifth ecdysis and pupation.

4. Hatch occurred four days after oviposition (time 0), and ecdyses of the remaining four larval instars were at 6.4, 8.4, 10.6, and 13.7 days; pupation occurred at 22.8 days. The larvae underwent molt sleep in which they did not feed, defecate, or move until ecdysis. Durations of molt sleep were from 14 to 25 hr for each of the larval-larval ecdyses. The fifth instar did not have a comparable stage.

5. Growth rate was highest for the first-instar larvae and slowest for the fifth instar, with weight-doubling times of 11.3, 17.6, 11.4, 17.3, and 21.2 hr for the first through fifth instar feeding periods, respectively, at 25° C.

6. Body wetting, found only in the last instar, was induced by the onset of the scotophase. Use of this event marker allowed the selection of a group of insects that were capable of pupating five days later within one hour of each other.

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STUDIES OF THE PROTHORACICOTROPIC HORMONE IN THE TOBACCO HORNWORM, *MANDUCA SEXTA*

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Kopeć (1917, 1922) first demonstrated that mature larvae of the gypsy moth, *Lymantria dispar,* were unable to undergo metamorphosis when deprived of their brains. Additional experiments led him to conclude that a brain hormone stimulated metamorphosis. In the 1940's this conclusion became established as part of the classical theory of insect development and metamorphosis as a result of similar observations including those of Caspari and Plagge (1935), Kühn and Piepho (1936), Plagge (1938), Wigglesworth (1940), Schneider (1942), and Williams (1946).

The first experimental evidence that the brain elicits development by stimulating the prothoracic glands (PG) was obtained in experiments performed on *Hyalophora cecropia* (Williams, 1947). A few years earlier Fukuda (1940) had identified the PG as the source of an active molting principle which was subsequently isolated (Butenandt and Karlson, 1954), named "ecdysone" (Karlson, 1956), and ultimately identified as a polyhydroxylated steroid (Huber and Hoppe, 1965). The brain-PG relationship has since been repeatedly documented (Wigglesworth, 1952; Kambysellis and Williams, 1971; Agui, 1975; Bollenbacher, Agui, Granger, and Gilbert, 1979). It forms one of the pillars of current insect endocrinology which views insect development as a progression of stages, each initiated during discrete periods of ecdysone production by the PG under the tropic stimulation of a brain hormone (prothoracicotropic hormone, PTTH).

In point of fact, a role for the brain in insect molting and metamorphosis had detractors even before it had advocates. Beginning with the observations of Conte and Vaney in 1911 and continuing with those of Kopeć himself (1912, 1922), the literature cites multiple examples of metamorphic development by brainless insect preparations at or beyond the late feeding period of larval life (Bounhiol, 1938; Fukuda, 1944; Ozeki, 1954; Ishizaki, 1972; Mala, Granger, and Sehnal, 1977; Meola and Adkisson, 1977). Perhaps no more remarkable observation of development by brainless insects exists than that of Judy (1972) who reported the ability of brainless larvae of the tobacco hornworm, *Manduca sexta,* not only to undergo a larval molt, but also to feed and complete metamorphosis to morphologically normal adults.

Early in our study of the developmental endocrinology of the tobacco hornworm we undertook a reexamination of the role of the brain in this insect's life cycle. An individual of this species in the penultimate instar faces four major developmental transitions before reaching adulthood: a) a larval molt to the fifth instar, b) the feeding-to-wandering transition at the conclusion of the fifth instar, c) the larval-pupal molt, and d) the pupal-adult molt. These steps share several common features. The beginning of each is accompanied by a detectable rise in the hemolymph ecdysone titer (Bollenbacher and Gilbert, unpublished observations cited in Riddiford and Truman, 1978; Bollenbacher, Vedeckis, and Gilbert, 1975; Kaplanis, Thompson, Yamamoto, Robbins, and Louloudes, 1966). Isolated abdomens in all the larval stages are unable to develop (Truman, 1972; Fain and Riddiford ,1976; Truman and Riddiford, 1974; Nijhout, 1976), but such preparations can be induced to molt or metamorphose by injections of ecdysone (Fain and Riddiford, 1976; Nijhout, 1976) or by implantation of active prothoracic glands (personal observations). Moreover, diapausing pupae can be caused to develop by injection of ecdysone, as can isolated pupal abdomens. These observations strongly suggest that each of the four transitions is dependent on ecdysone production by the PG—a conclusion consistent with the classical model of insect development. Yet the aforementioned work by Judy (1972) as well as the observations of Truman and Riddiford (1974), Nijhout (1976), and Wilson and Larsen (1974) suggest that some of these transitions may be partially or completely independent of the brain. In the experiments reported here we have reexamined the role of the brain in each of the four ecdysone-dependent transformations.

Materials and Methods

Larvae were reared at 25° C on an artificial diet, as described by Bell and Joachim (1976) and Truman (1972), under either short-day (SD, 12L: 12D) or long-day (LD, 17L: 7D) photoperiods. Time-of-day was arbitrarily referenced to lights-off at midnight (24:00 = 00:00). Larvae of the following types were segregated early in the photophase of each day: freshly ecdysed fourth-instar larvae and pharate fourths showing slipped head capsules, freshly ecdysed fifths, and fifths showing freshly exposed dorsal vessels. The timing of events in the life cycle was as described by Truman (1972) and Truman and Riddiford (1974) except that the first 24 hr of each stage was termed Day 1 rather than Day 0.

Neck-ligatures were placed between the head and prothorax of larvae and between the head and prothoracic spiracles of pupae within 6 hr after pupal ecdysis. Isolated abdomens of larvae wére obtained by placing ligatures across the first abdominal segment or between the metathorax and first abdominal segment. In all cases the portion anterior to the ligature was excised. Ligatures were of cotton-covered polyester (J. & P. Coats). Individuals were anesthetized with CO_2 prior to ligation and the surgical procedures.

Operations other than ligation were carried out on individuals immersed in insect Ringer's solution (Ephrussi and Beadle, 1936). Manipulations of the larval brain were performed through a small flap cut to one side of the midline just above the juncture of the cuticular sutures of the head capsule. The brain was easily located and gently drawn toward the incision by the entering bundles of trachea and nerves. These bundles were severed with micro-scissors and the brain withdrawn. Pupal brains were removed through a horizontal incision on the vertex of the head. Loose brains were completely withdrawn from the insect before reimplantation into a lateral recess of the head. Brain implantations into ligatured insects were either by way of a small incision in the dorsolateral aspect of the thorax or abdomen or through an incision in an abdominal proleg which was then closed by a ligature proximal to the incision. Implantations into pupae were through a small incision in the dorsum of the head. All surgical incisions except those in larval prolegs were sealed by apposition of the cut edges, thorough blotting of the region surrounding the incision, and application of a small drop of melted Tackiwax (Cenco). Less than 5% of individuals failed to survive the operations, and these typically died within 1 to 3 days. They have been excluded from the data presented.

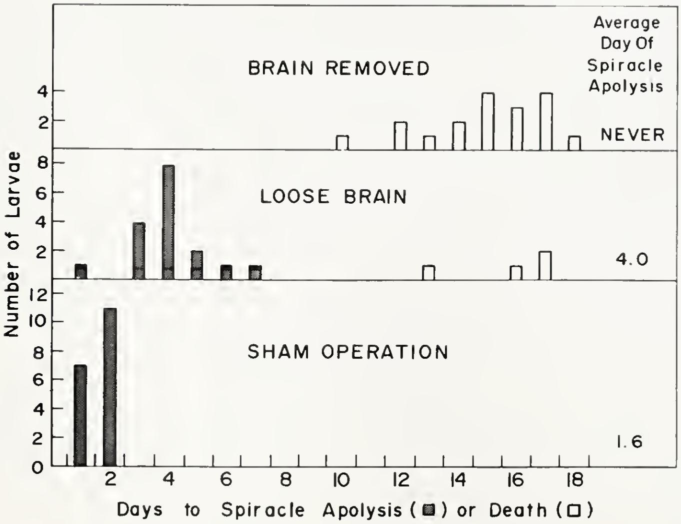

FIGURE 1. Inhibition of molting in fourth-instar larvae by operations involving the brain. Second-gate SD larvae were utilized at 20:00 to 23:00 on Day 3 of the instar. They were afterwards placed without food in individual plastic containers and examined daily for spiracle apolysis (solid box) or death (clear box). Unoperated larvae would typically have undergone spiracle apolysis on Day 1.

Two batches of partially purified PTTH from *Bombyx mori* (Professor H. Ishizaki of Nagoya University, Japan) were tested: one, "Crude PTTH," the second, "Highly-purified PTTH." The lyophilized extracts were dissolved for injection in 0.2 M ammonium acetate as suggested by Prof. Ishizaki. The extracts were tested within 1 week after being dissolved and were injected with a Hamilton syringe equipped with a 30 gauge needle. The biological activity of these extracts was ascertained by injection of an appropriate dose into brainless *Samia ricini* pupae.

The juvenile hormone analog (JHA) Hydroprene (ZR-512, Zoecon Corp.) was used. The compound was of technical grade and was dissolved in reagent grade acetone (Fisher) for topical application.

RESULTS

Molt of fourth-instar larvae

To analyze the role of the brain in larval molting we performed a series of operations on fourth-instar larvae. Second-gate SD larvae were segregated as previously described (Truman, 1972). All operations were performed at 20:00 to 23:00 on Day 3, following which the larvae were returned to individual plastic containers without food.

The results (Fig. 1) show that the brain is indispensable for the molt from the fourth to the fifth instar. Brainless larvae neither molted nor showed any other developmental response although they typically survived for 1.5 to 2.5 weeks. In additional experiments performed on dozens of individuals, no second-gate larva deprived of its brain prior to the third night of the instar ever underwent any further development.

When the brain was removed and then reimplanted into the head, the ability to undertake a larval molt was restored to many but not all individuals after a delay of several days. Autopsy of the larvae completing the molt failed to show reestablishment of any nerve connections except in two individuals where connections appeared to have been reestablished with the subesophageal ganglion or a corpus cardiacum.

When abdomens were isolated from fourth-instar larvae during the above-mentioned 3-hr period, no development took place in any of over 50 preparations. So also, no development was observed in 14 such abdomens receiving brain implants from larvae of the same age.

In another set of experiments, brains were removed from 51 second-gate larvae approximately 12 hr later than in the previous set of experiments—i.e., approximately 10 hr prior to the anticipated apolysis of the head capsule. All individuals underwent a larval molt. The majority experienced considerable difficulty in freeing their heads from the old head capsules; even when assisted in doing so, the head usually was sufficiently deformed in the area of the mandibles as to prevent feeding. Nevertheless a total of 11 of these brainless larvae underwent normal ecdysis and resumed feeding after they were restored to diet. Six of these pupated successfully after a variable delay and of these all ultimately completed adult development as previously described by Judy (1972).

Feeding-wandering transformation of fifth-instar larvae

When second-gate larvae were neck-ligated at times beginning shortly after 00:00 on Day 5, the normal onset of the wandering stage on Day 6, signaled by exposure of the dorsal vessel, occurred on schedule or with little delay. If the brain played a role in the transformation of these larvae into wanderers, the critical period for its action must have been virtually concluded by early on Day 5. For this reason two groups of fifth-instar larvae prior to Day 5 were utilized in a series of operations examining the brain's role at the feeding-to-wandering transition. One group contained second-gate LD larvae weighing 6.5 to 8.5 g and was operated on between 19:00 and 22:00 on Day 4 of the instar. The other group consisted of 4.0 to 4.5 g LD larvae and was operated on early on Day 3 of the instar. All larvae were returned to plastic containers without food following the operation.

The results (Fig. 2) show that the absence of a brain caused a substantial delay in the time to dorsal vessel exposure, this effect being especially pronounced in the smaller individuals. Indeed, many of the larvae in the latter group never exposed the dorsal vessel although they survived for over 3 weeks. Reimplantation of a brain into brainless larvae lessened but did not fully eliminate the delay occasioned by brain removal.

When abdomens were isolated from larvae similar to either of the above groups, no development took place during the 2 to 3 weeks of survival. Nor was development provoked in any of 16 abdomens isolated from larvae of the older class when a brain from individuals of the same age was implanted.

Wandering larva-pupa transformation

A series of operations was performed on 60 SD wandering larvae at 12:00 to 15:00 on the day of dorsal vessel exposure. Larvae were examined daily thereafter for pupation. Unoperated larvae pupated after 5.2 ± 0.4 days, sham-

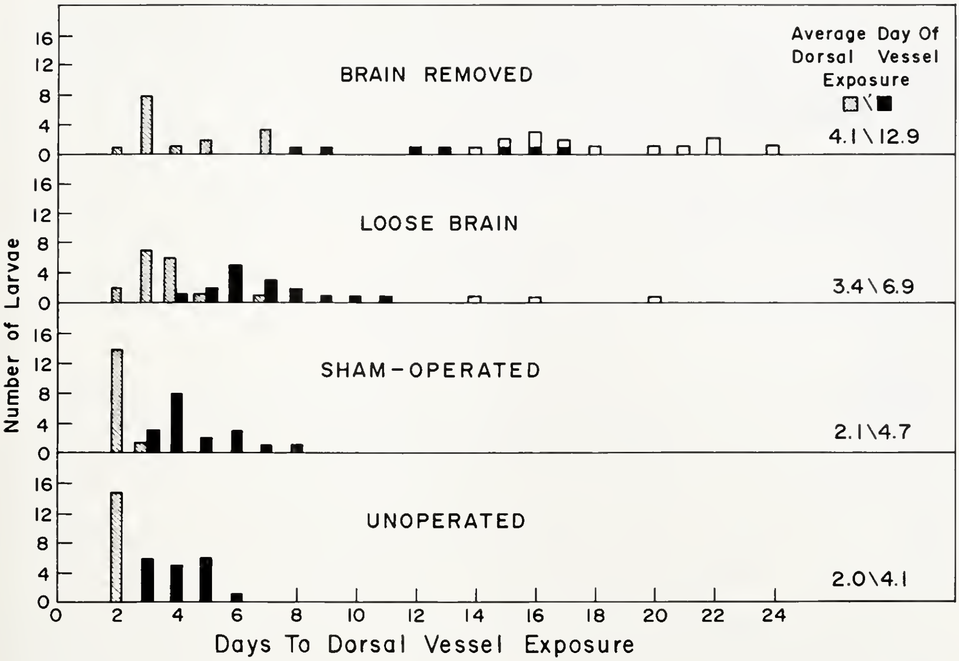

FIGURE 2. Inhibition of dorsal vessel exposure in fifth-instar larvae by operations involving the brain. LD larvae in two weight classes were utilized: 6.5 to 8.5 g larvae (hatched box) and 4.0 to 4.5 g larvae (non-hatched boxes). Larvae of the smaller class which died prior to dorsal vessel exposure are designated by clear boxes, those which exposed the dorsal vessel by solid boxes. Following operation, the larvae were returned to individual plastic containers without food and examined daily thereafter for dorsal vessel exposure or death. Averages were computed only for those larvae which exposed the dorsal vessel.

operated larvae after 6.0 ± 0.0 days, and brainless pupae after 7.2 ± 0.8 days. Wandering larvae lacking a brain were thus unquestionably delayed relative to both unoperated and sham-operated controls. As with the younger larvae, abdomens isolated from wandering larvae were unable to pupate. And when brains from Day 1 wandering larvae were implanted into 11 such abdomens, no development occurred during the subsequent 1.5 to 3 weeks of survival.

Pupal-adult transformation

To examine the role of the brain in the regulation of adult development and of pupal diapause, a series of operations was performed on LD and SD pupae during the 12 hr following pupal ecdysis. The results (Fig. 3) demonstrate the ability of the brain to provoke the initiation of adult development as signaled by apolysis of the wing epidermis. Both LD and SD pupae deprived of their brains experienced a substantial delay before initiating development—far beyond that observed in intact, diapausing pupae. The reimplantation of loose brains greatly curtailed but did not fully eliminate this delay. Loose SD brains stimulated development only after a latent period similar to that encountered in normal diapausing pupae; this was so irrespective of whether the host was a SD pupa or a brainless LD pupa. Loose LD brains uniformly stimulated prompt development and appeared to function with identical effect in either LD or SD hosts.

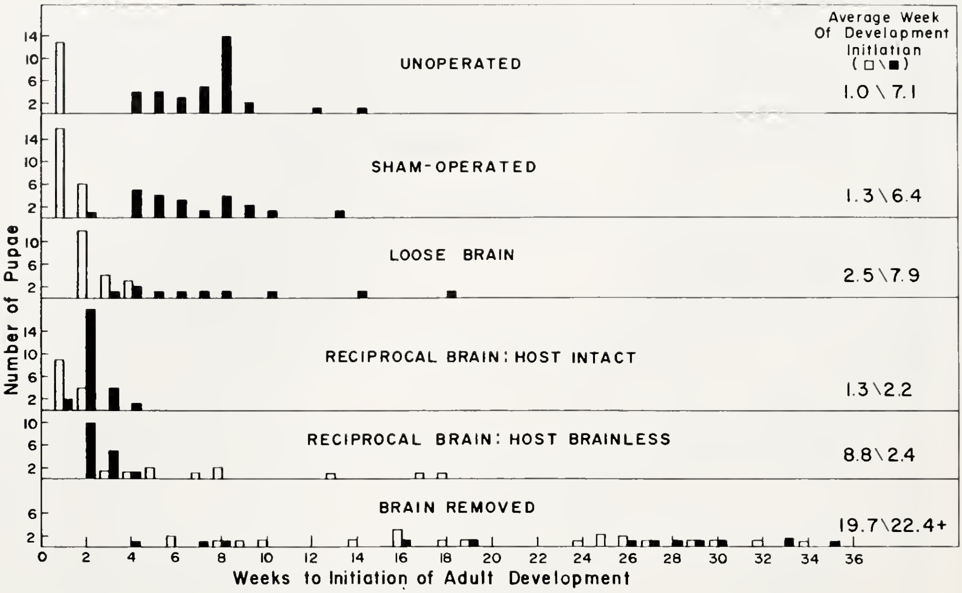

FIGURE 3. Initiation of adult development following operations involving the pupal brain. Diapause-destined SD pupae (solid box) and non-diapausing LD pupae (clear box) were utilized within 12 hr of pupation and examined weekly thereafter for initiation of adult development as signaled by apolysis of the wing epidermis. Sham-operated pupae were incised and the brain visualized but not touched. In the case of reciprocal brain implants, a brain from a pupa of the alternate photoperiod was implanted into a normal or brainless pupa; in the figure the graphic designation indicates the diapause program of the pupal host. In the case of brainless SD pupae, 4 individuals had not yet initiated development at 36 weeks.

In all cases development once initiated proceeded to completion in the usual period of about 18 days.

Identity of the brain tropic factor throughout the life cycle

We tested the ability of larval and pupal brains to cause the molting of larvae and the adult development of pupae. The larval brains were obtained from LD second-gate fifth-instar larvae between 07:00 and 12:00 on Day 5; the pupal brains from LD animals 6 to 12 hr after pupal ecdysis. These were implanted into diapausing pupae and into second-gate fourth-instar larvae head-ligated prior to 21:00 on Day 3 of the instar; the latter were topically treated with 20 μg of JHA to permit larval molting. The host pupae would normally have undergone development after an average of 1 to 2 months, while the larval hosts would normally have undergone no development whatsoever. Development of the host was recognized by initiation of apolysis in pupae and by spiracular apolysis in larvae. The results of these experiments were unambiguous (Table I). Larval as well as pupal brains caused the molting of larvae and the adult development of pupae.

Localization of prothoracicotropic activity in the nervous system

Several ganglia from Day 5 second-gate fifth-instar larvae were examined for their ability to elicit the development of diapausing pupae: these included the

TABLE I

Effects of implanted larval and pupal brains on the development of larval and pupal hosts.

Donor	Host	Number of assays	Percent developing	Time to initiation of development (days)
Larva	Pupa	12	100	10
Pupa	Pupa	15	100	9
Larva	Larva	10	80	4*
Pupa	Larva	13	69	5*

* Averages computed only for developing individuals.

brain, the subesophageal, prothoracic, mesothoracic, and terminal abdominal ganglia. Epidermis was implanted as a control. Single implants were made into diapause-destined pupae 6 to 18 hr after pupal ecdysis. Each type of ganglion was assayed in 15 to 17 pupae, except for the mesothoracic ganglion, which was assayed in 8 pupae. Activity was measured by averaging the time to initiation of development by the assay pupae, determined by apolysis of the wing epidermis. Only the brain possessed any significant tropic activity. Pupae receiving brains initiated development an average of 1.5 weeks later. Groups of assay pupae receiving other ganglia took from 5 to 7 weeks to initiate development and did not differ significantly from pupae receiving an implant of epidermis, which took an average of 6 weeks.

Assay of Bombyx mori *PTTH extracts*

We attempted to demonstrate a prothoracicotropic effect of *Bombyx mori* PTTH extracts in larvae and pupae of *Manduca sexta*. For assay of this activity we used 3-week old diapausing pupae, 1-week old diapausing pupae whose brains had been removed on the day following ecdysis, and fourth-instar larvae whose brains had been removed prior to PTTH release. Both crude and highly-purified PTTH preparations were inert in each of these bioassays at concentrations up to 100 *Samia* units, the highest dose tested.

DISCUSSION

Brain removal significantly delayed the development of larvae and pupae when carried out at appropriate intervals prior to the initiation of ecdysone-mediated transformations. The molting of larvae and the onset of adult development in pupae were especially delayed. When loose brains were reimplanted into the head, the delay occasioned by brain removal was consistently reduced but not eliminated. Abdomens isolated from larvae at various stages never undertook development nor were they caused to develop by the implantation of active brains. These results are consistent with the classical view of the brain as the source of a hormone which stimulates ecdysone production by the prothoracic glands at each ecdysone-dependent transition.

Judy (1972) noted a larval-larval molt in only one exceptional individual among his many brainless fourth-instar larvae. The present results (Fig. 1) further document the absolute necessity of the brain for larval molting. Brainless fourth-instar larvae resumed development only after the brain had played its role or after restoration of an active brain.

In the case of pupae, both diapausing and non-diapausing individuals required a brain in order to initiate adult development on schedule. Removal of the pupal brain routinely delayed the initiation of development by weeks or months, while implantation of loose brains restored the development of brainless hosts to a nearly normal rate. Pupal brains demonstrated the remarkable ability to retain their original commitment to diapause or not to diapause even when detached from all connections. Thus, as shown in Figure 3, brains from non-diapause pupae triggered prompt initiation of development, whereas brains from diapause-destined pupae caused development only after a delay typical of such brains *in situ*. Retention of normal endocrine behavior by loose insect brains has been previously observed (Williams, 1946; Williams and Adkisson, 1964; Truman and Riddiford, 1970).

Remarkable, too, was the failure of the host environment to affect the diapause or non-diapause programs of implanted pupal brains. Whenever the host received a brain from a non-diapausing donor, the host initiated development promptly regardless of its own program; in turn, brains from diapause-destined pupae caused development of brainless LD hosts only after a delay whose duration was similar to that of a normal diapause. When diapause-destined brains were implanted into intact non-diapausing hosts, they did not deter the prompt development stimulated by the host's brain. Hence, the timetable for development appears to be programmed into the pupal brain. These data suggest that *Manduca* possesses a classical pupal diapause in that the developmental standstill is due to a temporary cessation of the brain's tropic action on the PG.

The sum total of the present evidence clearly indicates that the brain normally exercises a prothoracicotropic function at each ecdysone-mediated transition in the tobacco hornworm. But while removal of the brain prevented or greatly delayed the development of fourth-instar larvae and of pupae, its effects where much less dramatic in feeding or wandering fifth-instar larvae. Furthermore, the eventual development of brainless *Manduca* pupae contrasts with the classical examples of complete developmental arrest following brain removal in saturniids (Williams, 1946).

Exceptions to the current model of insect development can be grouped as contradictions to one or more of four assumptions implicit in the classical scheme: a) Only the PG produce ecdysone; b) Only the brain activates the PG; c) PG function is entirely dependent on stimulation by the brain and, consequently, fluctuations in PG activity reflect changes in the output of the brain's PTTH; d) The onset of development occurs only after a particular quantity of ecdysone has been secreted.

Though the production of ecdysone by the PG is no longer disputed (Chino, Sakurai, Ohtaki, Ikikawa, Miyizaki, Ishibashi, and Abuki, 1974; King and Marks, 1974; King, Bollenbacher, Borst, Vedeckis, O'Connor, Ittycheriah, and Gilbert, 1974; Romer, Emmerich, and Novock, 1974), other sources of ecdysone are obviously present in at least certain species. For example, ovarian synthesis of ecdysone has been documented in several species (Hagedorn, O'Connor, Fuchs, Sage, Schlaeger, and Bohn, 1975; Lagueux, Hirn, and Hoffman, 1977; Bollenbacher, Zvenko, Kumaran, and Gilbert, 1978). Moreover, ecdysone production and even ecdysone-dependent development have been observed in isolated abdomens and preparations lacking prothoracic glands (Chadwick, 1955; Nakanishi, Moriyama, Okauchi, Fujioka, and Koreeda, 1972; Hsiao, Hsiao, and DeWilde, 1975; Studinger and Willig, 1975; Gersch and Eibisch, 1977; Delbecque, Delchambre, Hirn, and DeReggi, 1978). The role of these other ecdysone sources during normal molting and metamorphosis remains an enigma. Nevertheless, the failure of isolated abdomens of tobacco hornworm larvae to undertake any development even when supplied with an active brain suggests that sources of ecdysone other than the PG are unlikely to account for the development of brainless individuals in the final larval stage of this species.

While the brain is certainly the chief source of tropic input to the PG, the literature documents several potential mechanisms for PG activation in the absence of the brain. Injury has been demonstrated to stimulate pupal development (McDaniel and Berry, 1967; Wilson and Larsen, 1974), but in the present study sham operations to larvae or freshly ecdysed *Manduca* pupae tended to delay rather than accelerate development. PTTH activity has been identified outside the brain of some insects in the corpora cardiaca, the corpora allata, or elsewhere (Ichikawa and Nishiitsutsuji-Uwo 1959; Gersch and Stürzebecher, 1968; Ishizaki, 1969; Gibbs and Riddiford, 1977). Moreover, the corpora allata could potentially stimulate ecdysone production by virtue of their ability to produce juvenile hormone (Williams, 1959; Gilbert and Schneiderman, 1959; Hiruma, Shimada, and Yagi 1978). Nevertheless, in the present study bioassays of several ganglia failed to disclose significant PTTH activity in the nervous system outside the brain of *Manduca sexta*—a result in keeping with the findings of Gibbs and Riddiford (1977). Moreover, larvae and pupae lacking both brains and CA can undergo metamorphic development (Judy, 1972; Truman and Riddiford, 1974; personal observations).

Bombyx PTTH failed to elicit development of appropriate assay animals in the present study even though doses up to 100 times the amount necessary to wake up brainless *S. ricini* pupae were employed. Similar interspecific failures have been noted previously in studies using intact brains as well as brain homogenates as PTTH sources, although other such attempts have met with success (Gibbs and Riddiford, 1977; Williams, 1946; Ichikawa and Nishiitsutsuji, 1951). Assay of active brains from *Manduca* larvae or pupae demonstrated that both could accelerate ecdysone-dependent development in larval as well as pupal preparations (Table I). These data agree with prior results: for *Manduca* by Gibbs and Riddiford (1977), for *H. cecropia* by Williams (1952), and for *B. mori* by Kobayashi, Yamazaki, and Kimura (1973). Though the detailed chemistry of PTTH may vary between species, no convincing evidence supports the existence of more than a single native form within a particular species.

In the absence of a significant prothoracicotropic factor outside the hornworm brain, the PG might still secrete varying amounts of ecdysone if in the course of metamorphosis they exhibited stage-specific rates of spontaneous ecdysone production. Alternatively the PG might be subjected to stage-specific levels of inhibition. Hinton (1953) suggested that the PG might be inhibited as well as stimulated, but little clear evidence presently supports this possibility. An inhibitory factor has been supposed to originate variously from the brain (Carlisle and Ellis, 1968), from the subesophageal ganglion (Alexander, 1970), or from the thoracic ganglia (Mala, Granger, and Sehnal, 1977). The evidence is at best fragmentary and at times contradictory. Nevertheless the existence of a PG inhibitor arising outside the brain and variously employed during the life cycle remains an attractive explanation for the different rates of development by brainless individuals in the present experiments.

While molting and metamorphosis unquestionably require exposure to particular amounts of ecdysone, the relationship between the quantity of ecdysone necessary for a developmental response and the quantity produced is by no means a direct one. To trigger a developmental response the requisite amount of ecdysone must be produced, appropriately metabolized, and accumulated to effective intracellular concentrations. Furthermore, the visible start of development may be preceded by so-called "covert effects" (Ohtaki, Milkman, and Williams, 1968). Breakdown of ecdysone counters production, and even the covert effects of ecdysone may undergo decay (Ohtaki, Milkman, and Williams, 1968; Zdarek and Fraenkel, 1970). A small change in any one of these variables could stagger the rates of development of two individuals with identical rates of ecdysone production. Such a difference could well account for the contrasting behaviors of brainless cecropia pupae, which never develop (Williams, 1946), and brainless hornworm pupae, which finally develop after latent periods varying from weeks to months. Manifestly, the same considerations could influence the rates of development of brainless specimens of *Manduca* in the several developmental stages.

Removal of the brain unmasks aspects of ecdysone secretion, metabolism, and action which are normally hidden by the dominant rate-determining activity of PTTH. We have considered four assumptions underlying the classical model, one or more of which must be violated for brainless individuals to develop. The present data suggest that in *Manduca* only the PG produce ecdysone in amounts and at periods relevant to molting and metamorphosis. Moreover, the results here cannot readily be accounted for by the existence of prothoracicotropic factors originating outside the brain. The proper explanation of our findings therefore presumably lies in some combination of the following: changes in the autonomous activity of the PG; changes in the degree of inhibition of the PG; changes in ecdysone metabolism; changes in the sensitivity of the epidermis to ecdysone. As we shall demonstrate subsequently, several of these mechanisms seem in fact to have an important bearing on the development of the tobacco hornworm. But as the story unfolds, the reader must not forget that in every stage of development the brain plays a front and center role. While the present investigation suggests the possible existence of additional mechanisms of regulation, its results on the whole pay tribute to the brain of Stefan Kopeć.

This work was supported by grants from the National Science Foundation and the National Institutes of Health. We acknowledge the generous gift of partially purified preparations of PTTH and of brainless *Samia ricini* pupae from Prof. Ishizaki of Nagoya University, Japan. We also acknowledge the gift of the JHA Hydroprene by the Zoecon Corp.

SUMMARY

1. Brain removal at appropriate times in the life cycle uniformly delayed or prevented the onset of all known ecdysone-mediated transitions. This effect was especially pronounced at larval-larval molts and at the pupal-adult transformation.

2. Loose brains reduced but did not eliminate the delay induced by brain removal.

3. Isolated larval abdomens never developed nor were they induced to do so by implantation of an active brain.

4. Loose pupal brains retained their original commitment to diapause or not

to diapause. This was true even when the loose brain was implanted into a brainless host pupa with an opposite diapause commitment.

5. Larval brains effectively elicited the development of pupae, and conversely, pupal brains elicited the development of larvae.
6. Prothoracicotropic activity was found in the brain but in no other ganglia.
7. Extracts of *Bombyx mori* PTTH were inactive in *Manduca* in the concentrations tested.
8. Consideration is given to several mechanisms which might mediate the development of brainless hornworms.

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THE MORPHOLOGY, LIFE-HISTORY, AND TAXONOMIC RELATIONS OF *LEPOCREADIUM AREOLATUM* (LINTON, 1900) STUNKARD, 1969 (TREMATODA: DIGENEA)[1]

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It has long been known that the hydrozoan and scyphozoan medusae and ctenophores of the Woods Hole area harbor unencysted metacercariae of digenetic trematodes. In an abstract, Stunkard (1967) reported natural infections in *Bougainvillia carolinensis, Gonionemis vertens,* and *Chrysaora quinquecirrha.* Infections were found also in ctenophores, *Pleurobrachia pileus, Beroe cucumis,* and *Mnemiopsis leidyi.* He (1978b) reported five species of metacercariae in the ctenophores and medusae of the region. Three species have been identified and their life-cycles have been worked out. Descriptions of the successive stages in the intermediate and final hosts are published: that of *Neopechona pyriforme* (Linton, 1900) by Stunkard (1969b); of *Lepocreadium setiferoides* (Miller and Northrup, 1926) Martin, 1938 by Stunkard (1972); and *Lintonium vibex* (Linton, 1900) Stunkard and Nigrelli, 1930 by Stunkard (1978a). The asexual generations and life-cycle of a fourth species are described in the present report.

MATERIALS AND METHODS

The metacercariae were found first on 3 August 1973 in specimens of *Mnemiopsis leidyi,* taken in Buzzards Bay, off North Falmouth, Massachusetts. But the identity of the species was quite unknown. Moreover, at least five species of metacercariae were common in the ctenophores and medusae of the area and at that time the life cycles of only two, *Neopechona pyriforme* and *Lepocreadium setiferoides,* had been elucidated. The discovery in 1978 of an ophthalmotrichocercous cercaria in *Nassarius trivittatus,* whose structure agreed with that of the metacercaria, first collected in 1873, led to the resolution of the life-history. Incidence of infection by this species is very slight; the cercaria had not been found in the examination of thousands of specimens of *N. trivittatus.* Only one infected snail was found in 1978 and only one in the examination of hundreds of snails in 1979. The adults produce very few, very large eggs, (Figures 8–11). It should be noted that *N. trivittatus* harbors the asexual stages of another digenetic trematode; an unidentified hemiurid species.

The cercariae are produced in rediae in the haemocoele of the snail. Haemal sinuses contain rediae of various sizes and maturing cercariae (Figure 1). The presence of daughter rediae demonstrates more than one generation of rediae. The cercariae leave the redia in very immature condition, when the eye-spots are circular, pin-head units, and the tail is a non-motile appendage. They mature in the haemocoele and shortly before emergence, setae appear on the tail. They leave the snail through the gills, during the night or early morning and swim at all

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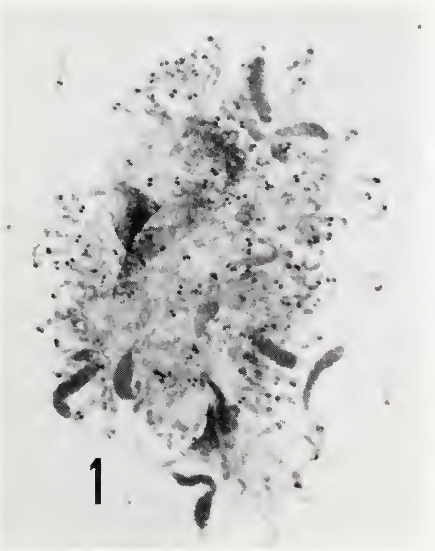


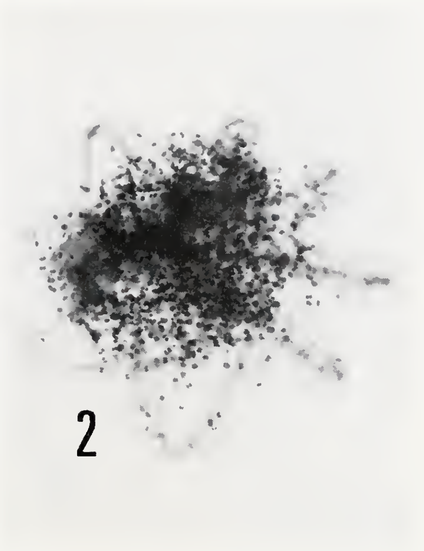
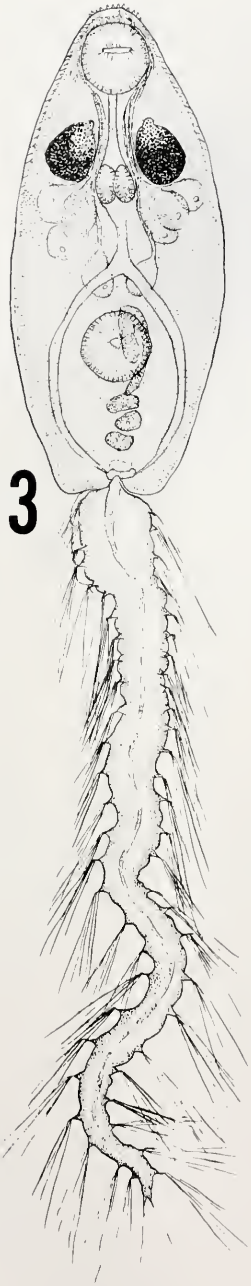


FIGURE 1. Microphotograph, haemal sinus from a specimen of *N. trivittatus,* with immature rediae and cercariae.

FIGURE 2. Microphotograph, intense infection of *Pleurobrachia pileus* exposed two hours to cercariae from *N. trivattatus.*

levels in the water. In swimming, the body is contracted, bent ventrally, the tail is extended and lashes so fast that only a blur appears. They tend toward the light side of the container. They swim with the tail in advance and when contact with a medusa or ctenophore is made, the tail adheres and the cercaria turns to bring the anterior end of the body in position for attachment. By movements of the tail and body, the body penetrates the jelly and the contents of the penetration glands are extruded. When the body is firmly embedded, the activity of the tail frees it from the body and it swims away. When a specimen of *Beroe cucumis* was placed in a bowl with swimming cercariae, they entered the grooves beside the comb-rows, penetrated, and migrated in the jelly. They did not penetrate the pigmented areas between rows. As the cercariae age, they move lower in the water and eventually sink to the bottom. The snail collected at Quissett on 20 July 1978 was shedding and continued to liberate cercariae every day until it was killed on 2 September to get the rediae and developing cercariae, (Figure 1). The snail taken 7 June 1979 was shedding and it continued until 20 July, after which no more cercariae appeared, although it was kept in cool running water and fed daily. It was crushed and examined on 17 August 1979 and contained about sixty rediae but no cercariae, either in the haemocoele or in the rediae. The liver was moribund, lacking in firm consistency and typical cellular organization, and the rediae contained only germ balls. It is well known that when a host is not properly nourished, trematode reproduction is retarded or ceases, and remains dormant until the host recovers its health and vigor. In this instance, it appears that the tissues of squid, clams, hermit crabs and small fishes, fed to the snail, were lacking in some vitamin or other substance essential for the complete metabolic requirements of the snail.

For experimental infection, it is essential to have hosts that are known to be free of previous exposure, or to produce massive infections that can not be miscon-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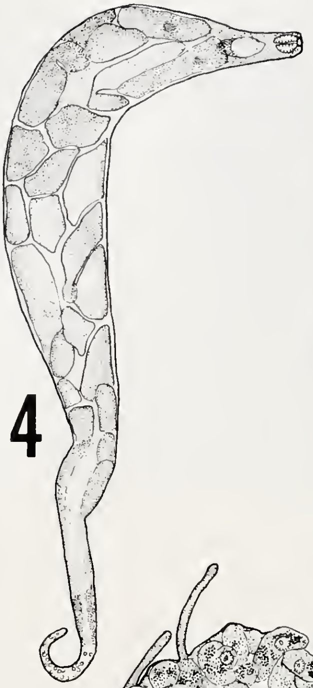
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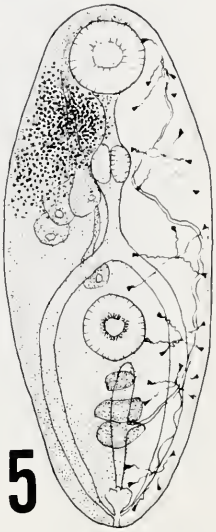
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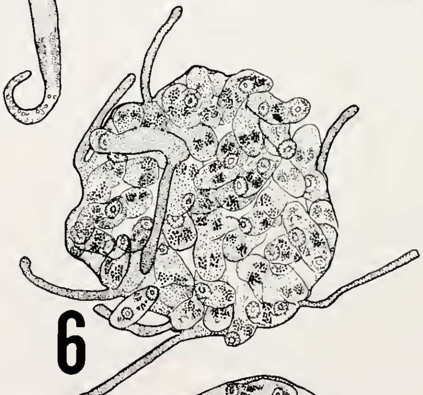
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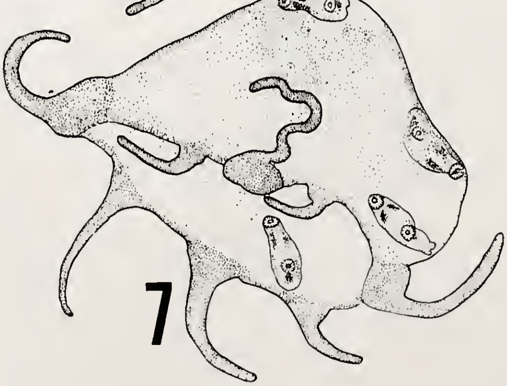
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FIGURE 3. Cercaria from *N. trivittatus*, fixed, stained and mounted.
FIGURE 4. Redia from *N. trivittatus*, fixed, stained and mounted.
FIGURE 5. Metacercaria from *Mnemiopsis leidyi*, composite drawing from sketches of living specimens, showing penetration glands and details of the excretory system.

strued (Fig. 2). The ctenophores and medusae from plankton collections contain metacercariae which presage the possibility of error in subsequent interpretation. Crowell (1945) reported that *Podocoryne carnea* lives on the shells of *N. trivittatus,* especially on shells that are occupied by hermit crabs. A unique ecological situation obtains in nature; the snail harbors the trematode which liberates cercariae; the hydroid liberates medusae, which are attacked and penetrated by the cercariae (Figs. 6 and 7). Medusae of *P. carnea,* produced in the laboratory and known to be free of infection, were exposed to cercariae from *N. trivittatus,* and later, embedded in bits of squid or clam, were fed to cunners, *Tautogolabrus adspersus,* that had been maintained for about one month in the laboratory, during which time any natural infections would have matured. Fed twice a week, the method yielded juvenile and gravid specimens of *Lepocreadium areolatum,* a species described initially from white perch and cunners by Linton (1900, 1901), and known also from puffers, *Spheroides maculatus,* and other fishes.

RESULTS AND DESCRIPITIONS

The Adult. (Figures 8–11).

Fixed, stained and mounted specimens may be 1.4 mm long and 0.60 mm wide but most are less than 1.00 mm in length. The largest of the four specimens from the cunner, taken by Linton in 1899 and deposited in the U. S. National Museum under the number 8280, is shown in Figure 10. It is 1.10 mm long and 0.50 mm wide. The worms become mature at a much smaller size and the one from a puffer, shown in Figure 8, with only two eggs, is 0.40 mm long and 0.22 mm wide. The one egg that has a shell, is 0.12 by 0.07 mm; the same size as eggs in larger specimens. In this species the acetabulum is situated about one-third of body length from the anterior end and is somewhat smaller than the oral sucker. Measurements of the holotype were given by Stunkard (1969a). Other specimens are depicted in Figures 9 and 11.

The redia. (Fig. 4).

The rediae are cylindrical, attenuated at both ends, with a curved, pointed, tail-like posterior region. They vary in size from small individuals, 0.20 by 0.04 mm, with germinal cells and a few forming germ-balls to others 1.60 mm long and 0.20 mm wide, filled with germ balls and developing cercariae. The pharynx is spherical to oval, typically longer than broad, 0.048 to 0.058 mm long and 0.030 to 0.040 mm wide. The caecum is small, slightly larger than the pharynx, but displaced and inconspicuous when developing cercariae impinge on the pharynx.

The cercaria. (Fig. 3).

The cercariae are distomate, ocellate, and trichocercous. The body is oval to ovate, somewhat pointed anteriorly. Fixed and stained specimens average 0.38 mm long and 0.16 mm wide. In living specimens the body may be longer and narrower or shorter and wider. The acetabulum is about 0.05 mm in diameter,

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FIGURE 6. Medusa of *Podocoryne carnea,* intense infection after exposure two hours to cercariae from *N. trivittatus.*

FIGURE 7. Medusa of *Podocoryne carnea,* slight infection, with four metacercariae of *Lepocreadium areolatum.*

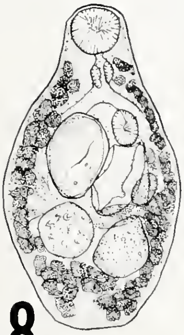
situated in the posterior half of the body; the distance from the anterior end to the acetabulum is about twice as long as the distance from the acetabulum to the posterior end. The opening of the sucker is equipped with a double row of small alternate spines. The tail is 0.45 to 0.54 mm long, 0.05 mm wide at the base, and tapers distally. There is a row of nuclei along the median line and several near the tip. There are twenty-seven pairs of setae and a terminal tuft. The lateral setae have a dorso-ventral alignment and arise from bulb-like expansions of the wall of the tail. They are 0.08 to 0.10 mm long; the terminal ones somewhat shorter. In the anterior fourth of the body the tegument is spined. The ocelli are 0.05 mm long and 0.035 mm wide, situated obliquely, so that the anterior tips are directed mediad. They are separated by about the width of an ocellus. There are four pairs of penetration glands, whose ducts stain with neutral red. One pair is median, below and slightly posterior to the intestinal caeca. Their ducts pass forward under the caeca. There are three pairs anterior and lateral in position; the ducts from the penetration glands pass on the median sides of the ocelli and open near the anterior tip of the body. The oral sucker is subterminal longer than wide and 0.05 to 0.06 mm in diameter. The prepharynx is about as long as the diameter of the oral sucker. The pharynx, situated between and behind the ocelli, is 0.035 mm in diameter. The esophagus, lined with epithelium, is about as long as the prepharynx and the bifurcation of the digestive tract is near midbody, anterior to the acetabulum. The caeca are long and communicate by short, narrow ducts with the posterior end of the excretory vesicle. The intestinal contents stain with neutral red, and when a stained, living specimen is pressed under a coverglass, pink material from the caeca is extruded through the excretory pore. Details of the excretory system were observed in the cercariae and metacercariae. The flame-cells and capillaries in the region of the oral sucker and pharynx were studied in the cercaria, while the ocelli are intact; dispersal of pigment in the metacercaria obscures the excretory structures in this area. The excretory pore is closed by a strong sphincter and the vesicle extends forward to the level of the acetabulum. Collecting ducts open into the vesicle about midway between the acetabulum and the sphincter. The ducts pass forward, turn laterad at the level of the acetabulum under the caeca and continue to the level of the ocelli. Here they turn posteriad and receive a branch from the region of the oral sucker, another from the postoccular area, and a third from the preacetabular region. There each receives the duct which drains the postacetabular area. There are six sets of five cells, as shown in the figure of the metacercaria and the flame-cell formula is $2[(5+5+5)+(5+5+5)]$.

The metacercaria. (Figure 5).

In the ctenophores and medusae, which according to Joyeux and Baer (1961, p. 451) serve as "hôtes d'attente ou paraténique," the cercariae mature but do not increase much in size. The ocelli disintegrate and the pigment is dispersed. Growth occurs primarily in the posterior half of the body; the gonads which are very small in the cercaria, double in size. In the posterior half of the body the flame-cells and capillaries are resolved more readily in the metacercaria than in the cercaria, but the pattern does not change.

Systematic Relations and Discussion

Linton (1900) identified specimens from the white perch, *Morone americana*, taken 27 August 1898 at Woods Hole, as *Distomum areolatum* Rudolphi, 1809.



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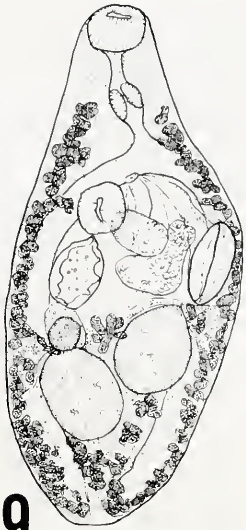

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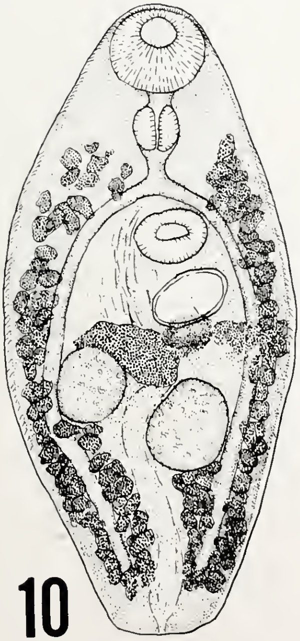

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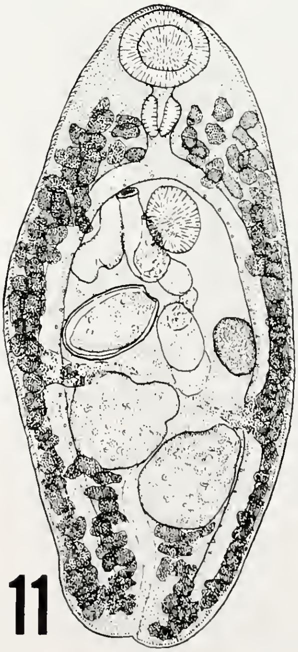

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FIGURE 8. *Lepocreadium areolatum*, specimen from *Spheroides maculatus*, 0.40 mm long.

FIGURE 9. *L. areolatum*, specimen from *Spheroides maculatus*, 0.92 mm long.

FIGURE 10. *L. areolatum*, from *Tautogolabrus adspersus*, specimen number 8280 in the U. S. National Museum, 1.10 mm long.

FIGURE 11. *L. areolatum*, from *T. adspersus*, experimental infection, 0.74 mm long.

The worms were found in a dish in which the viscera of the fish had been lying, and the determination was admittedly doubtful. The description was supplemented by Figure 60, Plate 39, and a representative specimen, stained and mounted, was deposited in the U. S. National Museum under the number 6517. The description was based on the mounted specimen, whose measurements were given. Linton (1901), without description or figure, identified, p. 462, specimens taken 5 August 1899 from the cunner, *Tautogolabrus adspersus,* and p. 486, specimens taken the same date from the winter flounder, *Pseudopleuronectes americanus,* as *Distomum areolatum* Rudolphi. The specimens from the cunner were deposited in the U. S. National Museum under the number 8280.

In a report on parasites of fishes taken in July and August, 1901 and 1902, at Beaufort, North Carolina, Linton (1905) identified as *Distomum areolatum* Rud., specimens from *Bairdiella chrysura, Micropogon undulatus, Orthopristis chrysopterus,* and *Sciaenops ocellatus.* The descriptions are too sketchy to have taxonomic value, but the report of infection in *B. chrysura* was supplemented by three figures.

In a posthumous publication, Linton (1940) reviewed, redescribed and reallocated the trematodes from fishes, mainly from the Woods Hole region, that he had assembled in the previous more than 40 years. In the genus *Lepocreadium* Stossich, 1904, he recognized three species: (1) *Lepocreadium pyriforme* (Linton, 1900) described originally as *Distomum pyriforme* n. sp., from the rudderfish, *Palinurichthys perciformis;* (2) *Lepocreadium retrusum* n. sp., from the chub mackerel, *Pneumatophorus grex;* and (3) *Lepocreadium trullaforme* n. sp., from the long-spined sculpin, *Acanthocottus octodecimspinosus,* and other fishes.

Linton's description of *Lepocreadium trullaforme* n. sp., comprised worms collected at different times and from various species of fishes. It included specimens from the long-spined sculpin, *A. octodecimspinosus;* four worms taken 26 April 1915, including the holotype, were deposited in the U. S. National Museum under the number, 8276. Although a specimen from *A. octodecimspinosus* was designated as holotype of *L. trullaforme,* that specimen was not figured; instead, Figure 54 of a worm from *Morone americana* and Figures 53, 55, and 56 of specimens from *Menticirrhus saxatilis* were used to illustrate the morphology of *L. trullaforme.*

In the new species, Linton included: (1) the specimens from *Morone americana,* described (Linton, 1900, p. 293, Figure 60, Plate 39, U. S. National Museum, number 6517, as *Distomum areolatum* Rudolphi; (2) two specimens from the American sole, *Achirus fasciatus,* reported as *Distomum* sp. in Linton (1901, p. 487, Figure 351, Plate XXXI) with types in the U. S. National Museum, number, 8275; (3) one specimen from the kingfish, *Menticirrhus saxatailis,* taken 11 September 1907, in U. S. Museum, number 8277; (4) many specimens, some immature, from young kingfish, *M. saxatilis,* collected 21 August 1918, in U. S. National Museum, number 8278; (5) eight specimens from the white perch, *M. americana,* collected 8 August 1910, in U. S. National Museum, number 8279; (6) specimens from the winter flounder, *Pseudopleuronectes americanus,* collected 5 August 1899, no description or figure, listed as *Distomum areolatum* Rudolphi in Linton (1901, p. 486); and (7) specimens from the cunner, *Tautogolabrus adspersus,* collected 5 August 1899, listed as *Distomum areolatum* Rudolphi (Linton, 1901, p. 462) in U. S. National Museum, number 8280.

Sogandares-Bernal and Hutton (1960) reviewed the genus *Lepocreadium;* they redescribed and figured *Lepocreadium trullaforme* from the holotype, but the host was given as the cutlassfish, *Trichiurus lepturus*, and the locality as Woods Hole, Massachusetts. They predicated, p. 277, "Linton (1940) pictured different species under the name *L. trullaforme*. We have examined Linton's material and find that he had identified at least two different species as *L. trullaforme*". They recounted the specific features of the species and described the single specimen from the kingfish, deposited in the U. S. National Museum under the number 8277, as a new species, *Lepocreadium caballeroi.*

In an earlier report, Sogandares-Bernal and Hutton (1959) described *Lepocreadium floridanus* n. sp., and devised a key to *Lepocreadium* and closely related genera. In *L. floridanus* the ovary is lobed. They noted, p. 58, "In connection with species of *Lepocreadium* with unlobed ovaries, *L. archosargi* and *L. micropogoni* are probably synonyms of *L. ovale*. A restudy of the type specimens of all three species would be necessary in order to confirm this point." Later, they (1960) examined these holotypes but apparently did not resolve the problem. It should be noted that *L. ovale* Manter, 1931; *L. archosargi* Pearse, 1949; *L. micropogoni* Pearse, 1949; like *L. caballeroi* Sogandares-Bernal and Hutton, 1960, were described from single specimens. The descriptions of *L. archosargi* and *L. micropogoni* as given by Pearse (1949) do not portray specific differences and the holotype of *L. micropogni* was reported by Sogandares-Bernal and Hutton (1960, p. 280) to be in such poor condition that they suggested it be regarded as *species inquirende*. They noted, p. 280, "*L. archosargi* appears to be closely related to *L. caballeroi*, but differs by possessing a cirrus sac which hardly extends posterior to the acetabulum, more anterior vitellaria, and by the presence of ani. Both *L. archosargi* and *L. caballeroi* share the distinction of having eggs which are longer than the acetabulum." *Lepocreadium archosargi* was distinguished from *L. ovale* by what are regarded as trivial and dubious differences. *Lepocreadium ovale* Manter, 1931 was based on a worm from the pinfish, *Lagodon rhomboides* at Beaufort, North Carolina, and as noted by Sogandares-Bernal and Hutton, is probably identical with the worms described by Pearse (1949).

The small worms from a young kingfish, 55 mm long, collected 21 August 1918, number 8278 in the U. S. National Museum, were redescribed by Linton (1940). He noted, p. 90, "A characteristic feature of these distomes is the large size and small number of the ova. No more than two ova were seen in any one of them. Some of the smallest were immature, and contained no ova, but ova were present in quite small specimens. Thus, one, length 0.28 mm., breadth, 0.27 mm., contained one ovum, 0.10 by 0.06 mm., another, length, 0.28 mm., breadth, 0.25 mm., contained two ova 0.10 by 0.05 mm., and 0.10 by 0.06 mm." These worms depicted on Plate 6, Figures 55 and 56, agree so completely with the description and figures of *Lepocreadium setiferoides* (Miller and Northup, 1926) Martin, 1938 as given by Stunkard (1972) that their identity is apparent. The morphology is supported by data from the life-history. Linton (1922) reported on the food of young winter flounders. After listing the secondary intermediate hosts of *L. setiferoides*, Stunkard (1972) observed, p. 329, "The nature of the secondary intermediate hosts probably explains the observations of Linton (1922) and Martin (1938) that infection occurs only in young and small fishes, since older fishes, six to eight inches in length, feed on other and larger organisms."

The specimens from *Morone americana* collected 8 August 1910, in the U. S. National Museum under number 8279, conform to the description of *Distomum areolatum* Rudolphi, collected from the same host-species, 26 August 1898.

The specimens from the winter flounder, *P. americanus,* taken 5 August 1899, without description of figure, were listed as *Distomum areolatum* in Linton (1901, p. 486) ; their identity is indeterminate.

Specimens from the cunner, *Tautogolabrus adspersus,* in the U. S. National Museum under number 8280, were included in *L. trullaforme* by Linton (1940). They were collected 5 August 1899 and assigned by Linton (1901, p. 462) to *Distomum areolatum* Rudolphi. There are four worms on the slide; the most representative one is shown as Figure 10 in this report.

It may be significant that the several worms, taken at different times from various species of fishes, and identified originally as *Distomum areolatum* Rudolphi, were assigned by Linton (1940) to the new species, *Lepocreadium trullaforme.* Sogandares-Bernal and Hutton (1960) demonstrated that they are not identical with *L. trullaforme,* but the possibility remains that some of them may be included in a common species. The first of these specimens, identified as *Distomum areolatum* Rudolphi, was taken 27 August 1898 from the white perch, *M. americana.* The description was based on a mounted specimen, depicted as Figure 60, Plate 39, and deposited in the U. S. National Museum under the number, 6517. It was restudied by Stunkard (1969a), who recognized it as a member of the genus *Lepocreadium* and designated it as type of *Lepocreadium areolatum* (Linton, 1900) n. comb., adopting the specific name proposed by Linton. It should be noted that the eggs were few and very large, 0.115 by 0.070 mm.

The species includes specimens of natural infection, taken by the writer over the years from cunners, *T. adspersus,* and from puffers *Spheroides maculatus* (Figs. 8 and 9) and after experimental infection of cunners (Fig. 11). The species with large eggs, *L. ovale, L. archosargi, L. micropogoni,* and *L. caballeroi* are very similar and specific identity is not excluded. Linton (1905) reported *Distomum areolatum* from *Orthopristis chrysopterus, Bairdiella chrysura, Micropogon undulatus,* and *Sciaenops ocellarus* at Beaufort, North Carolina. In each instance, the eggs were few and large, 0.120 by 0.070 mm. Linton's (1901, Fig. 346) of *Distomum* sp., which Manter assigned to *L. ovale,* agrees so completely with the description and figures of *L. areolatum* that specific identity is probable. Although the evidence is not final, there is reason to regard *L. ovale, L. archosargi, L. micropogoni,* and *L. caballeroi as* synonyms of *L. areolatum.*

SUMMARY

Digenetic trematodes from the white perch, *Morone americana,* were identified by Linton (1900) as *Distomum areolatum* Rudolphi, 1809. He (1901) referred specimens from the cunner, *Tautogolabrus adspersus,* and the winter flounder, *Pseudopleuronectes americanus,* to the same species. These worms were collected at Woods Hole, Massachusetts. Other specimens were taken from *Bairdiella chrysura, Micropogon undulatus, Orthopristis chrysopterus,* and *Sciaaenops ocellatus* at Beaufort, North Carolina. Linton (1940) included all these worms in a new species, *Lepocreadium trullaforme.* But they are not specifically identical with *L. trullaforme* and their taxonomic status has been uncertain. The metacercariae of these worms have been known for many years an unencysted distomes in the ctenophores and medusae taken in plankton collections. The discovery of an ophthalmotrichocercous cercaria from *Nassarius trivittatus* with the same morphology, has led to the completion of the life-cycle and the resolution of the taxonomic status of the species, which proved to be *Lepocreadium areolatum*

(Linton, 1900) Stunkard, 1969. Cercariae from *N. trivittatus* penetrated and matured in medusae of *Podocoryne carnea,* maintained in the laboratory. Experimentally infected medusae were fed twice a week to cunners, which had been isolated for more than a month, and a series of developmental stages from juveniles to gravid worms were recovered. All stages in the life-cycle are described and figured.

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3,4-DIHYDROXYPHENYLALANINE (DOPA) AND SCLEROTIZATION OF PERIOSTRACUM IN *MYTILUS EDULIS* L.

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The catecholic amino acid, 3,4-dihydroxyphenylalanine (DOPA), is present in specialized cells of most animals where it serves as an intermediate in the biosynthesis of catecholamine hormones, neurotransmitters and melanin pigments (Nagatsu, 1973).

A somewhat different role of DOPA is suggested by its occurrence in several invertebrate extracellular structural proteins including leech egg capsule (Knight and Hunt, 1974), trematode egg capsule (Rainsford, 1967), mytilid byssus fibers (Degens and Spencer, 1966) and the periostracum of various bivalve molluscs (Degens, Spencer and Parker, 1967; Waite, 1977; Waite and Andersen, 1978). All these structures share the property of being sclerotized by an undefined process of protein cross-linking termed quinone tanning (Brown, 1950; Brown, 1952). DOPA is the only known immediate precursor of quinones in these structures, but no experimental data exist about the role of DOPA in the protein cross-linking process.

In the periostracum of *Mytilus edulis,* DOPA is at all times an integral part of the structural protein and can not be dissociated from it without the complete hydrolysis of the protein. Waite and Anderson (1978) recently reported the recovery of significantly more DOPA from hydrolysates of new marginal periostracum than from older exposed periostracum. The apparent disappearance of DOPA might result from its modification by cross-linking to proteins, thus rendering the proteins less soluble with time, *i.e.,* sclerotization. To test this possibility, we have measured the amounts of DOPA and soluble protein in increasingly aged periostracal annuli in the shell of the mussel, *Mytilus edulis.*

MATERIALS AND METHODS

Live mussels were collected from Issefjord (Denmark) during the months May to July. The age of each specimen was estimated by counting superficial annuli, and this estimate was corroborated by examination of umbonal cross-sections (Lutz, 1976). The periostracum was scrubbed clean under running tap water and peeled from the shells after partial decalcification in aqueous acetic acid (4%) and methanol (2.5%). Peeled periostracum was rinsed in three changes of distilled water and cut into 5 or 6 annular sections (Fig. 1). The ledge of periostracum along the shell margin is simply referred to as marginal periostracum. Each of the 39 sections was dehydrated in 95% ethanol, freeze-dried, pulverized and weighed. Powdered sections were subdivided into two lots—one to be assayed for soluble protein and, the other, for the determination of DOPA and tyrosine after acid hydrolysis. Soluble protein was extracted from periostracum with formic acid (13.0 M) at 4° C for 24 hr. Insoluble protein

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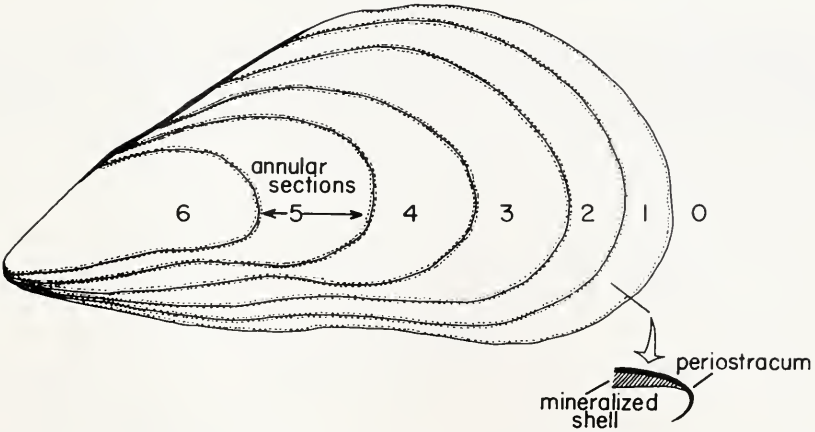

FIGURE 1. A drawing of the right valve of *Mytilus edulis* depicting the crescent-shaped annular sections into which the periostracum was cut. The insert (lower right) affords a cross-sectional view of the marginal periostracum. This is the only area where periostracum does not overlay mineralized shell. The numbers associated with the annular sections correspond roughly to the *average age* of a given section. Age is in years.

was removed by filtration through fritted glass; the filtrate was concentrated by rotary evaporation to a final volume of 2 to 4 ml. Aliquots of 2 to 4 μl or less were assayed for protein using a modified Lowry procedure (Hartree, 1972). Soluble protein from marginal periostracum was diluted 10-fold with distilled water, dialyzed against the same (12 hr at 4° C), freeze-dried and stored at −20° C.

Periostracal samples were hydrolyzed for 24 hr in 6 M HCl with 0.1% caprylic acid and 1.0% thiodiglycol at 110° C *in vacuo*. The acid was removed by vacuum evaporation at 50° C, and the residue was dissolved in 0.2 M acetic acid (2–3 ml) and centrifuged at 10,000 × *g* for 30 min to remove insoluble materials. Tyrosine and DOPA were separated as described previously (Waite and Andersen, 1978), and quantitated at 280 nm using the molar extinction coefficients 1180/(M·cm) [tyrosine] and 2600/(M·cm) [DOPA] (Merck Index, 8th Edition, 1968). Amino acid composition of three periostracal samples (soluble, marginal and first-annular periostracum) was determined after acid hydrolysis (described above) for 24, 48, and 72 hr on a Beckman Model 120 C analyzer according to the method of Moore (1968). Annuli older than one year were not analyzed for amino acids due to difficulty in insuring complete removal of attached extraneous marine flora and fauna.

Isolation of aromatic compounds other than DOPA and tyrosine from acid hydrolysates of periostracum (marginal) was performed according to the method of Waite and Andersen (1978) using gel filtration on Bio-Gel P-2 (50 × 1.5 cm) and ion exchange on cellulose phosphate in tandem. Eluants of each column were monitored for per cent transmittance at 280 nm. Isolated individual peaks were then scanned for absorbance between 225 and 325 nm with a Pye-Unicam SP-8000 spectrophotometer. Thin-layer chromatography on Merck cellulose Fertigplatten was used to check the purity of the isolated peaks. The solvent system was 4-parts 2-propanol to 1 part acetic acid to 1 part water, and the indicator was ninhydrin (0.1%) in absolute alcohol.

RESULTS

An apparent increase in the age of periostracum is accompanied by decreasing solubility of periostracal protein in formic acid and by decreasing concentrations of DOPA and tyrosine in hydrolysates of periostracum (Fig. 2). DOPA and

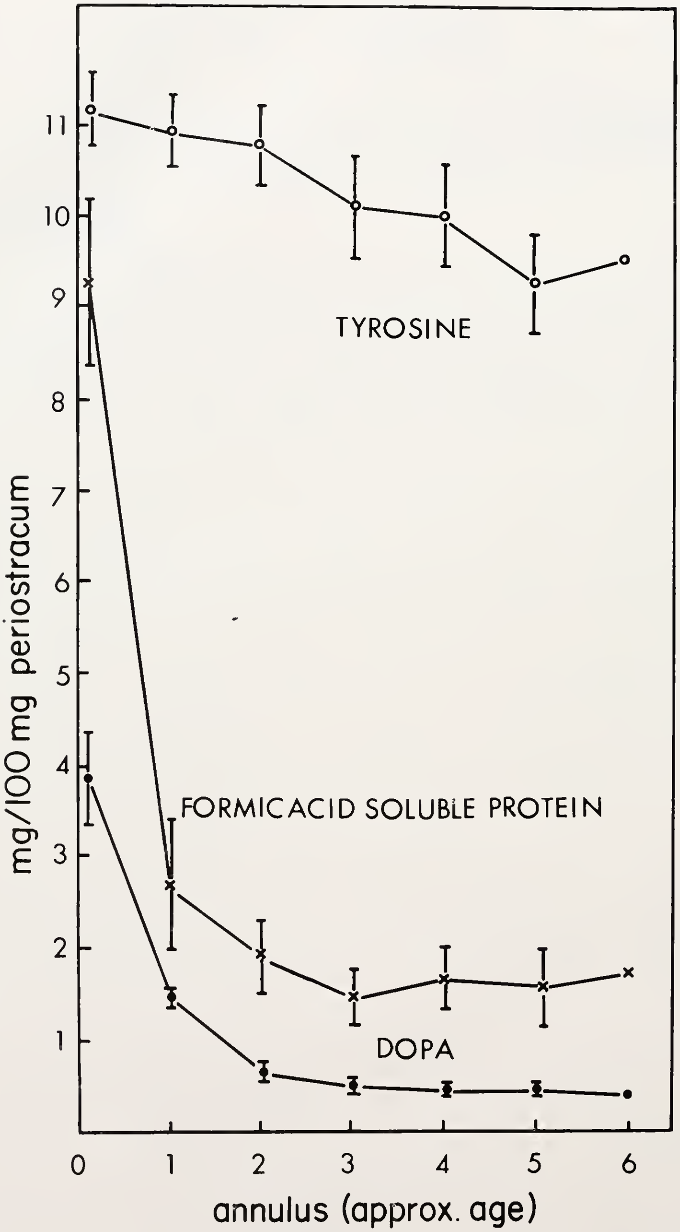

FIGURE 2. Variation of DOPA, tyrosine and protein solubility in periostracum with respect to the age of the annular section. Each point (excepting the last) represents the mean of 6 to 8 trials. Bars define the standard error of the mean. Numbers in the abscissa denote the *average age* of that annulus (years).

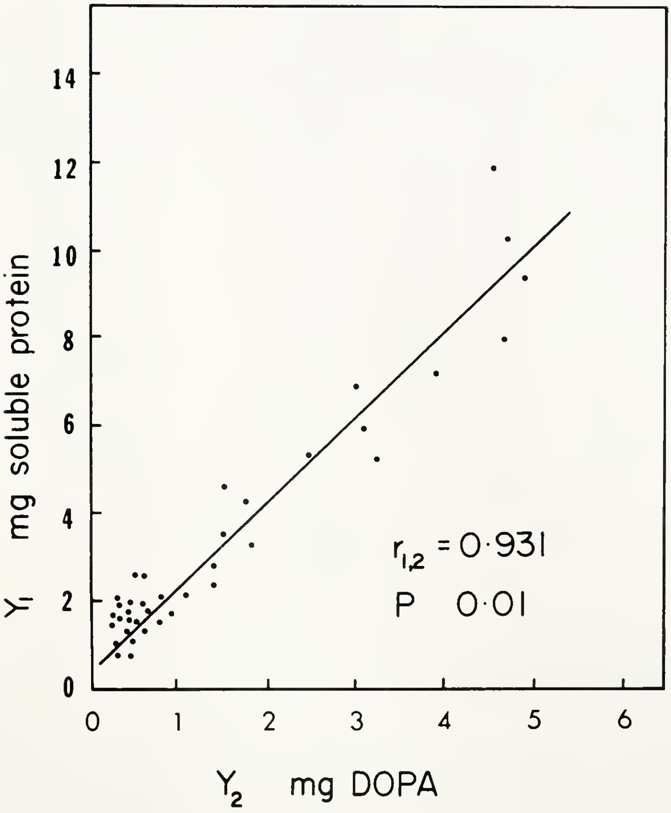

FIGURE 3. Plot of the correlation of DOPA and soluble protein in *Mytilus* periostracum. Dots represent 39 observations in which protein solubility (per 100 mg periostracum) in formic acid (Y_1) is plotted against its DOPA content (Y_2) determined after complete hydrolysis of the protein. The product-moment correlation coefficient is designated $r_{1,2}$ and was calculated according to Sokal and Rohlf (1969) from all the points in the distribution. P denotes probability that linear distribution is significant.

protein solubility exhibit decreases that resemble first order decay rates with asymptotes at 0.5 and 1.5% of weight, respectively. In contrast, tyrosine does not decrease significantly ($t_s = 1.323$, df = 11) between the margin and the first annulus; subsequently, its decrease is gradual and occurs throughout the life span of the periostracum.

Formic acid (13 M) is an effective protein denaturant and solvent, and for this reason, was chosen to extract all periostracal proteins not stabilized by covalent bonds. Figure 2 illustrates that, with the exception of the margin, very little protein can be extracted from periostracal annuli. The curves for protein solubility and DOPA in periostracum are sufficiently similar to suggest that a correlation exists between the two variables. Figure 3 shows a plot of all paired DOPA-protein solubility measurements. The data exhibit a roughly linear distribution ($R = 0.931$) and support the interpretation that a decrease in recoverable DOPA is directly proportional to decreasing protein solubility. The chief advantage of this correlation plot is that it eliminates the need for the most approximately measured variable, *i.e.*, age.

TABLE I

Amino acid composition of soluble, marginal and annular periostracum reported as percent weight of total weight.

	Soluble % (g/g)	Marginal % (g/g)	1st Annulus % (g/g)	1st Annulus / Margin
Asp	2.2	4.3	4.5	1.05
Thr*	1.4	1.2	1.0	0.83
Ser*	2.6	3.4	2.7	0.75
Glu	2.5	2.3	2.1	0.91
Pro	2.0	1.8	1.7	0.94
Gly	22.9	26.5	25.9	0.98
Ala	1.6	2.0	1.7	0.85
Cys/2	0.4	0.3	0.4	1.33
Val	2.5	3.3	3.3	1.00
Met	0.3	0.3	0.2	0.67
Ile	0.5	1.3	1.3	1.00
Leu	1.5	2.1	2.4	1.14
DOPA*	4.3	4.0	1.8	0.46
Tyr*	11.3	11.1	11.6	1.04
Phe	2.7	2.3	2.2	0.96
His	0.9	1.2	1.1	0.92
Lys	1.5	1.5	0.7	0.47
Arg	3.3	4.0	3.8	0.95
Total	64%	74%	68%	

* Corrected for losses due to hydrolysis.

Table I shows the per cent weight recovery of other amino acids from soluble, marginal and first annular periostracum and illustrates two significant points. The first is that formic acid-soluble protein from the periostracal margin is similar to the whole periostracum in amino acid composition. Both contain high concentrations of glycine and tyrosine; both contain DOPA. The other point in Table I is a comparison of the amino acid compositions of the margin and first annulus. In view of the drastic decrease in both DOPA and protein solubility between the margin and first annulus, we were particularly interested in determining what other, if any, amino acids decreased in the same interval. In agreement with Figure 2, results in Table I illustrate that tyrosine does not change significantly while DOPA is diminished by nearly 50%. Ignoring variation due to rounding off, most amino acids are as abundant in hydolysates of the first annulus as they are in the margin. Only lysine appears to be diminished by the same extent as DOPA, but the significance of this is difficult to assess because of the relatively low concentrations of lysine to begin with.

It is noteworthy that the weight recovery of periosracum after acid hydrolysis never approaches 100%. The balance of weight unaccounted for could consist of labile amino acids (tryptophan), sugars, pigments, residual water, lipids or mineral salts. A substantial yet still undefined portion of the periostracal hydrolysate appears to exist as ultraviolet light-absorbing aromatic material. In addition to the amino acids phenylalanine, tyrosine and DOPA, which are the first three peaks (A-C) to elute from a column of Bio-Gel P-2 (Fig. 4), there are at least seven other distinct peaks. D is known to be tryptophan, and H to be 6-bromotryptophan (Andersen, unpublished results). The apparent ability of some tryptophan to resist destruction during the acid hydrolysis of periostracum is not understood. Of the remaining peaks, E and G have been further char-

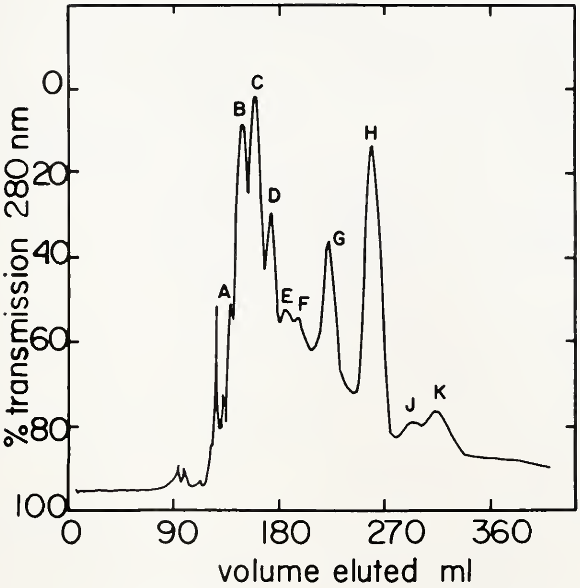

FIGURE 4. Elution of UV-absorbing compounds from acid hydrolysed marginal periostracum (50 mg) on a column of Bio-Gel P-2. Buffer is 0.2 M acetic acid and the flow rate is 12.5 ml/hr. Fractions containing peaks E and G were pooled for further purification by ion exchange on cellulose phosphate.

acterized and may shed some light on the fate of DOPA. E and G elute 2 and 6 hr, respectively after DOPA on cellulose phosphate using an ion gradient of 0.4 to 1.0 M NaCl according to Waite and Andersen (1978). On cellulose thin-layer chromatography, E and G migrate as uniform ninhydrin-positive spots with R_f values of 0.48 and 0.55, respectively, compared to 0.31 for DOPA. The ultraviolet spectra of the compounds between 225 to 325 nm, showed λ_{max} for both at 250 nm, and shoulders at 279 (E) and 284 nm (G). These spectra resemble strikingly some of the spectral characteristics of DOPA addition compounds with cysteine (Ito and Prota, 1977; Agrup, Hansson, Rorsman, Rosengren and Rosengren, 1976), and further research is expected to resolve whether these compounds might be DOPA-derived cross-links.

DISCUSSION

Insolubility, in addition to mechanical strength and resistance to proteolysis, is a key characteristic of scleroproteins (Linderstrøm-Lang and Duspiva, 1936). In the periostracum of *Mytilus edulis,* only newly secreted marginal material contains a substantial amount of protein soluble in formic acid; the remainder is almost wholly insoluble in the same solvent. Thus, it would appear that sclerotization occurs rapidly in *Mytilus.* Our results indicate that the degree of sclerotization of periostracal protein is closely correlated to the concentration of L-DOPA in acid hydrolysates of the protein.

Few will contest the premise that DOPA contributes to quinone-tanning. It is an immediate quinone precursor by enzymatic catalysis or auto-oxidation (Mason, 1948), and DOPA quinones can condense with amino acid residues to form

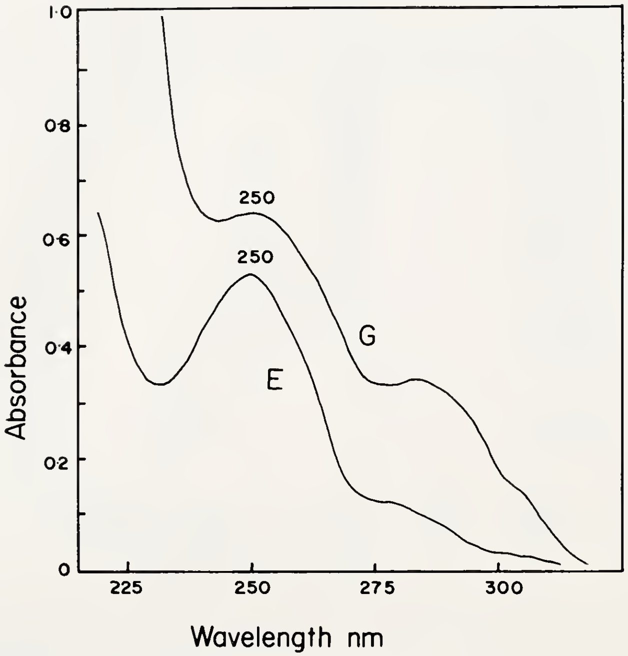

FIGURE 5. UV-spectra of purified peaks E and G. Maximal absorbance occurs at 250 nm. Solvent was 0.2 M acetic acid.

quinone adducts (Mason and Peterson, 1965; Ito and Prota, 1977). A problem invariably arises, however, with respect to whether tyrosine or DOPA actually serves as the precursor for quinones. Many of the current assumptions about sclerotization have been influenced by the studies of Lissitzky, Rolland, Reynaud and Lasry (1962) showing that DOPA transiently present in proteins *in vitro* is derived from tyrosyl residues by the direct action of mushroom polyphenoloxidase. Without compelling evidence, Pujol (1967) compared this reaction to the sclerotization of tyrosine-rich protein in *Mytilus* byssus. Similarly, many workers have since relied on the same reaction (often called autotanning) to explain sclerotization of tyrosine-rich proteins in molluscan periostracum.

We consider the mushroom polyphenoloxidase reaction as inadequate for describing sclerotization of periostracum. The following evidence now exists supporting a direct role of DOPA, not tyrosine, in the sclerotization of mytilid periostracum: it is known that periostracal phenoloxidase does *not* catalyze the oxidation of tyrosine (Waite and Wilbur, 1976); that catecholic compounds (DOPA) are abundant in the periostracum-secreting cells of the mantle (Bubel, 1973); that DOPA is present at proportions of up to 4% in a soluble presclerotin (MW 20,000) of formic acid-extracted periostracum (Waite, Saleuddin and Andersen, 1979), and that DOPA, not tyrosine, decreases during the major period of sclerotization between the margin and first annulus. Of course, DOPA still owes its existence to a post-translational modification of some tyrosyl residues by o-hydroxylation (Waite, Saleuddin and Andersen, 1979), but this event probably occurs during cellular processing of the protein, prior to its involvement in sclerotization.

Hunt and Oates (1978) have avoided the DOPA-tyrosine question by emphasiz-

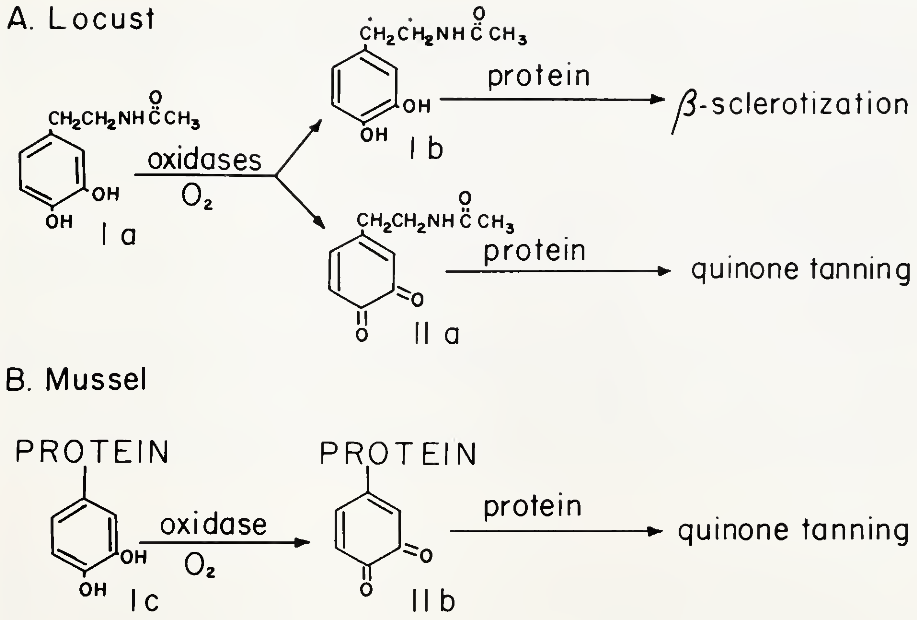

FIGURE 6. Sclerotization in locust cuticle (A) and *Mytilus* periostracum (B). Asterisks in A signify side chain carbons activated during β-sclerotization. Structures Ia-c are catechols: II a–b are quinones.

ing the products of aromatic cross-links in molluscan periostracum rather than the precursors. Unfortunately, nothing is known about the fate of DOPA following its oxidation to quinones in periostracum. One would assume that DOPA-quinones react with certain amino acid residues to form intermolecular cross-links. Lysine may be such a reactive residue, but our results are not yet conclusive about this. The observation by Morrison, Steele and Danner (1969) that quinones *in vitro* are capable of reacting with almost all amino acids could portend great difficulty in the isolation of specific cross-links from periostracum.

Sclerotization of exoskeletal proteins has been exploited throughout the animal kingdom, producing structures with a diversity of physical properties appropriate to the needs of the organism. A comparison of sclerotization of locust cuticle with that of *Mytilus* periostracum reveals striking similarities as well as differences. In both, the oxidation of catecholic precursors mediates protein sclerotization. However, in the locust cuticle (Fig. 6-A), the catechol, N-acetyldopamine, is of low molecular weight and freely diffusable. During sclerotization, it can be converted to either a quinone or a catechol with activated side chain (β-sclerotization). Thus, in the locust, sclerotization can be modulated by the local concentration of N-acetyldopamine relative to protein, and by the ratio of quinone-tanning to β-sclerotization (Andersen, 1974). In *Mytilus* periostracum (Fig. 6-B), protein with tightly bound DOPA residues is secreted from the mantle and cross-linked as the oxidized DOPA quinones react with available nucleophilic groups. Although less sophisticated than sclerotization in locust cuticle, the DOPA protein seems ideally suited to sclerotization of *Mytilus* periostracum; it is water-insoluble, reactive as a monomer, inert as polymer, frugal (catechol and protein in one) and can be secreted continuously without the need for exuviation.

Thanks are due to Mr. Tim Housley for his assistance in analyzing the amino acid composition and to the National Science Foundation (Grant SMI 76–17904) for financial support.

SUMMARY

1. The periostracum of *Mytilus edulis* is a highly sclerotized protein structure as demonstrated by its low solubility in 13 M formic acid.

2. The concentration of DOPA in periostracal hydrolysates is not uniformly distributed, but varies directly with protein solubility and logarithmically with the approximate age of the material.

3. The results suggest that the disappearance of DOPA is linked to the sclerotization of periostracum, probably by way of quinone-tanning; tyrosine does not appear to participate in sclerotization.

4. Sclerotization of DOPA-protein in *Mytilus* periostracum is compared to a similar process in the exoskeleton of the locust, *Schistocerca gregaria*.

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MORPHOLOGY OF THE CORTICAL REACTION IN THE EGGS OF *PENAEUS AZTECUS*

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Egg cortical reactions initiated by sperm penetration or exposure to sea water have long been known in many animal ova. Cortical reactions exist in the eggs of cnidarians (Dewel and Clark, 1974), annelids (Pasteels, 1965), arthropods (Hudinaga, 1942), echinoderms (Afzelius, 1956), teleosts (Yamamoto, 1939; Kagan, 1935), amphibians (Osanai, 1960), and mammals (Austin and Amoroso, 1959; Szollosi, 1962; Austin, 1965 and 1968, for review).

Dehiscence of the cortical granules at the periphery of a typical ovum into the surrounding media often causes the lifting of an egg investment coat with the resultant formation of a fertilization membrane (Endo, 1961a, b). It is believed that these cortical reactions are responsible for the prevention of polyspermy by both a chemical and physical block (Epel, 1975 for review) and that they also may establish a microenvironment inside a tough chorionic membrane for the developing embryo (Allen, 1958).

Changes in the cortices of animal ova have been studied extensively in the echinoderms, annelids, and amphibians. At present, however, there is very little information concerning egg activation in crustaceans. Since decapod crustacean fertilization is often internal, it is difficult to observe reactions of the egg. Hinsch (1971) reported the formation of a membrane around the eggs of *Libinia emarginata* that might be the result of a cortical reaction. Cheung (1966) has reported membrane formation probably initiated by fertilization around the eggs of *Carcinus*.

Work on the ovaries and the cortical structure in eggs of animals of the natantia is limited. Hudinaga (1942) briefly described a massive cortical reaction in the eggs of *Penaeus japonicus* and *P. monoceros*. In both animals, regions of "jellylike substance" were described in the peripheral cytoplasm of the eggs. These peripheral regions were released at spawning and formed a jelly coating around the surface of the egg that remained until the second cleavage.

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Preliminary reports (Clark and Lynn, 1977; Lynn and Clark, 1975; Clark, Lynn and Yudin, 1975) have described the cortical reaction in the eggs of *P. aztecus* and *P. setiferus*. The purpose of this paper is to carefully document the morphological events that occur in the cortex of *Penaeus* sp. ova upon contact with sea water.

MATERIALS AND METHODS

Using a standard otter trawl, gravid brown shrimp (*P. aztecus*) were collected 80 to 100 miles south of Galveston, Texas, and gravid white shrimp (*P. setiferus*) were caught 1 to 10 miles off the coast. Observations reported here, including figures, concern *P. aztecus*. Findings were confirmed with *P. setiferus*.

Animals were transported to the laboratory in a 150-gal aerated tank held at 15 to 20° C. Once in the laboratory, the gravid shrimp were placed in aerated, inverted, 5-gal carboys, and the water temperature was raised slowly to 28° C to induce spawning.

For transmission electron microscopy, eggs were fixed for 1 to 2 hr in a 0.1 M, PO_4-buffered (pH 7.5) paraformaldehyde-glutaraldehyde solution, with sucrose added to adjust osmolarity (Karnovsky, 1965). Samples then were post-fixed in 0.1 M, PO_4-buffered osmium tetroxide for 30 min. Fixed samples were dehydrated rapidly in a graded acetone series, embedded in a low-viscosity epoxy resin (Spurr, 1969), and sectioned with either glass or diamond knives on a Porter Blum MT2-B ultramicrotome. Thick plastic sections for light microscopy were stained with 1% toluidine blue buffered in 0.15% sodium borate (Dewel and Clark, 1974). Thin sections were stained with saturated methanolic uranyl acetate, counterstained with lead citrate (Venable and Coggeshall, 1965), and examined on a Hitachi HS-8 electron microscope.

For scanning electron microscopy, eggs prior to and at various stages of activation were fixed in a paraformaldehyde-glutaraldehyde solution as previously described, dehydrated in a graded acetone series, and critical-point dried. Samples then were coated with approximately 20 nm of gold and examined on a Cambridge S4-10 Stereoscan microscope.

RESULTS

Light microscopy of the cortical reaction

A mature, unreacted oocyte spawned directly into fixative is spherical and approximately 266 μm in diameter (Fig. 1A). Light striations, which represent cortical specializations (cortical rods), are apparent in the peripheral cytoplasm. When an oocyte is spawned into sea water, these cortical rods are released into the surrounding media. Figures 1A to 1L represent a series of light micrographs illustrating this reaction.

Early stages of the reaction are stopped for observation (Figs. 1B, 1C) by holding a spawning female over a beaker of sea water and fixing the collected eggs at 10 and 20 seconds, respectively. In these micrographs (1B, 1C), the rods have just begun to emerge from the egg and result in a blebbed appearance around the periphery of the egg. Figures 1D to 1L illustrate one egg undergoing a cortical reaction.

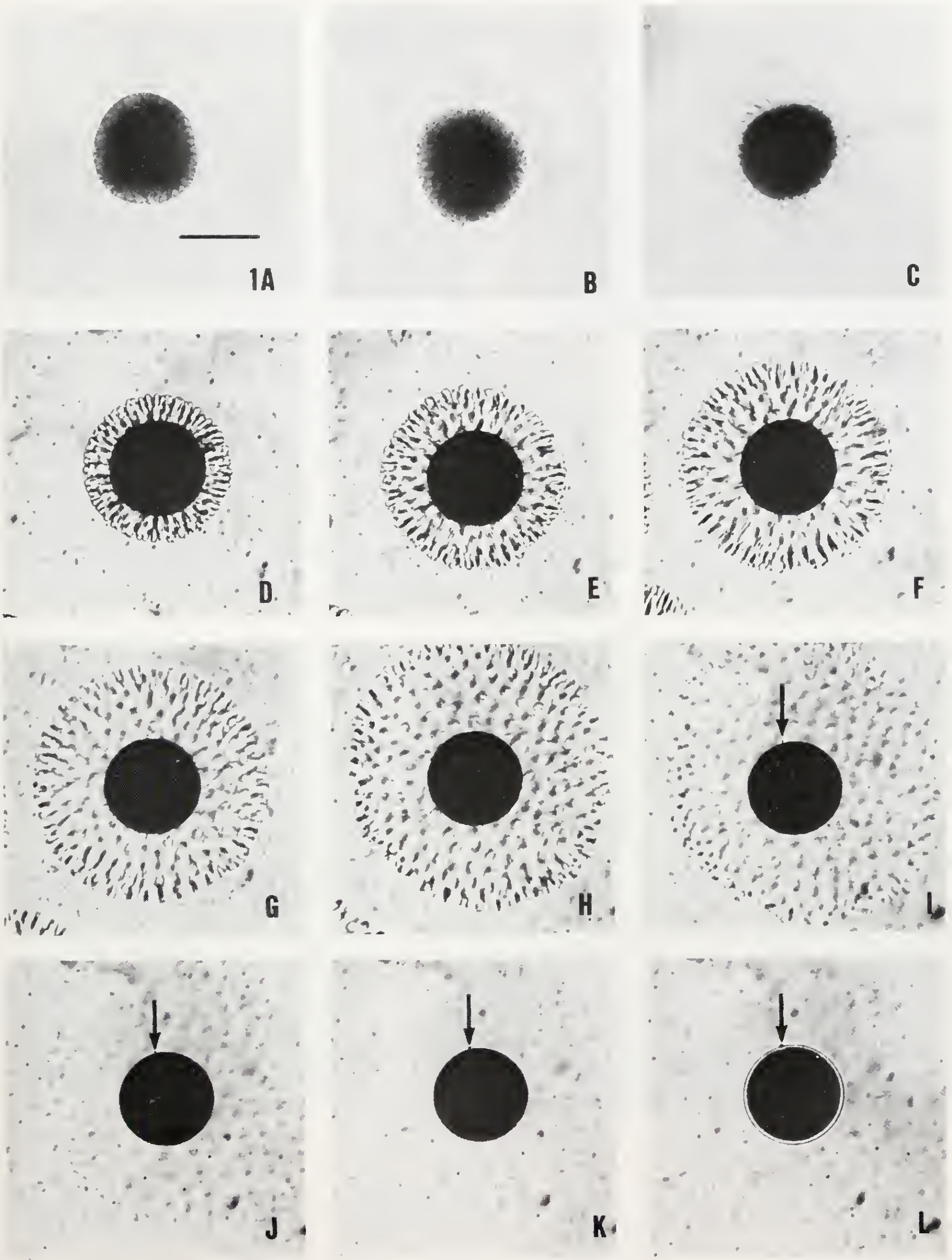
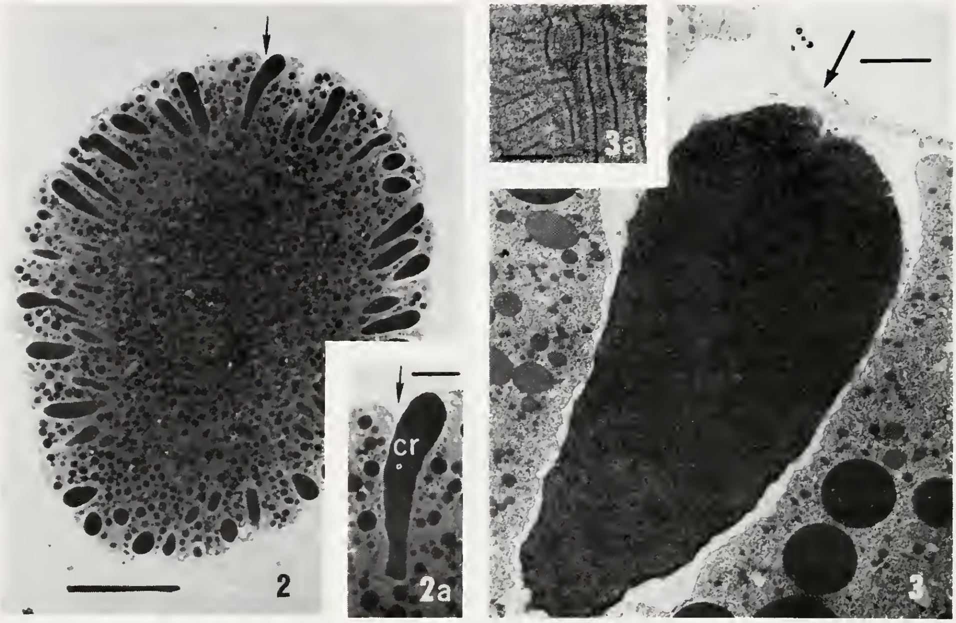
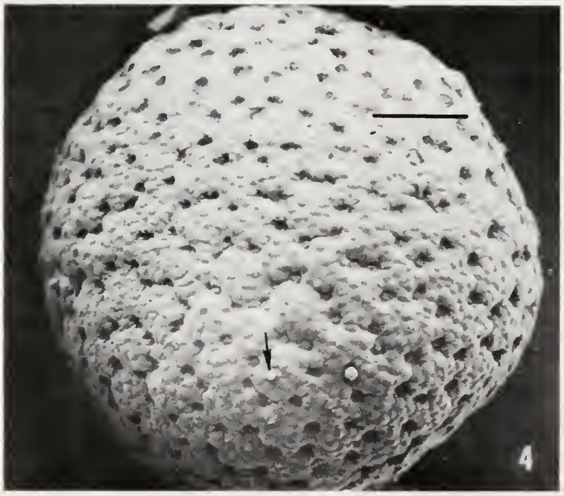


FIGURE 1A–1L. Light micrographs showing the cortical reaction of *P. aztecus* eggs from the unreacted stage, through the release of the polar body (arrow), to the formation of a hatching membrane. Bar = 200 μm.

Once the cortical rods are expelled, they form a corona around the egg (Fig. 1E). This corona remains as the rods swell and begin to dissipate (Figs. 1G, 1J). Within 5 to 7 min after initiation of the reaction, the cortical rods





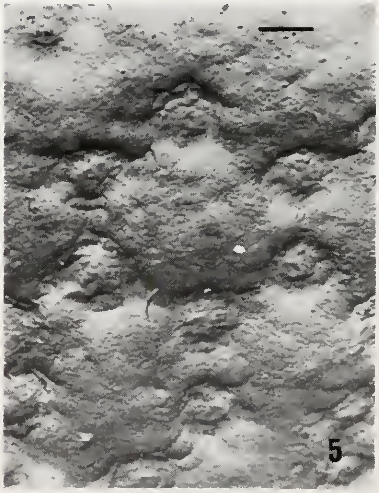


FIGURE 2. A light micrograph of a thick plastic section of an unreacted oocyte. Arrow points to the investment coat. Bar = 50 μm.

FIGURE 2a. A higher magnification showing the investment coat (arrow), which separates the club-shaped cortical rod (CR) from the external media. Bar = 10 μm.

FIGURE 3. A transmission electron micrograph of a cortical rod (CR) within its crypt. The investment coat is indicated by arrows. Bar = 2 μm.

FIGURE 3a. A high magnification revealing the fibrillar substructure of the cortical rods. Bar = 0.2 μm.

FIGURE 4. Scanning electron micrograph of an unreacted oocyte. Indentations in the investment coat represent the location of the cortical rods' crypts. A sperm attached to the investment coat is also shown (arrow). Bar = 50 μm.

FIGURE 5. A higher magnification revealing the indentations arranged hexagonally on the surface of the egg. Bar = 5 μm.

are dissipated completely (Fig. 1K). After cortical rod dissipation, a hatching membrane begins to form and is readily apparent after 15 min (Fig. 1L).

To facilitate a complete morphological description of the above events, the cortical reaction is divided into four stages. These stages are: unreacted stage; early reaction; corona stage; and dissipation stage. The formation of the hatching membrane is not discussed in this paper.

Unreacted stage

As noted previously, to examine unreacted oocytes, the eggs were spawned directly into fixative. The oocytes contain extensive accumulations of yolk platelets dispersed throughout the cytoplasm (Fig. 2). Large, conspicuous, "club"-shaped cortical rods are located in the peripheral cytoplasm (Figs. 2, 3). Each cortical rod is approximately 39.9-μm long with a basal diameter of 4.6 μm and an apical diameter of 9.3 μm (Fig. 2A). The rods lie within membrane crypts and are separated from the external media by a thin fibrous investment coat, 0.13-μm thick, that encompasses the egg (Figs. 2, 3). The substructure of the rods is distinct, each rod being composed of numerous, tightly packed, feathery elements (Fig. 3A). Each element is composed of a dense axis, 9-nm wide and of variable length, with lateral fibrillar projections that are 3.5 nm wide and 31.5 nm long (Fig. 3A). The overall structure of each element is reminiscent of a "bottle brush." When unreacted oocytes are viewed with the scanning electron microscope (SEM), hexagonal patterns of cortical rod crypts are revealed as indentations in the investment coat (Figs. 4, 5). Occasional sperm also are noted on the surface of the investment coat (Fig. 4).

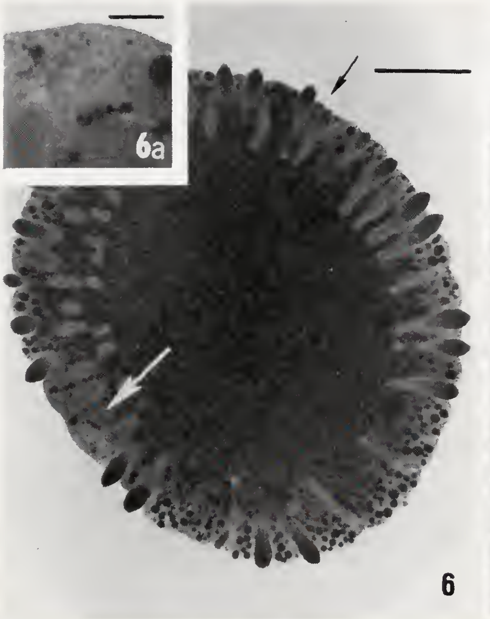
An unreacted oocyte is approximately 266-μm in diameter, or 9.85×10^6 μm^3 in volume, with a surface area of 2.2×10^5 μm^2. An oocyte has 746 ± 60 crypts. Assuming that each crypt is conically shaped, 39.9 μm long, and with an apical diameter of 9.3 μm and a basal diameter of 4.6 μm, the approximate volume and membrane surface area of each crypt can be determined (Table I).

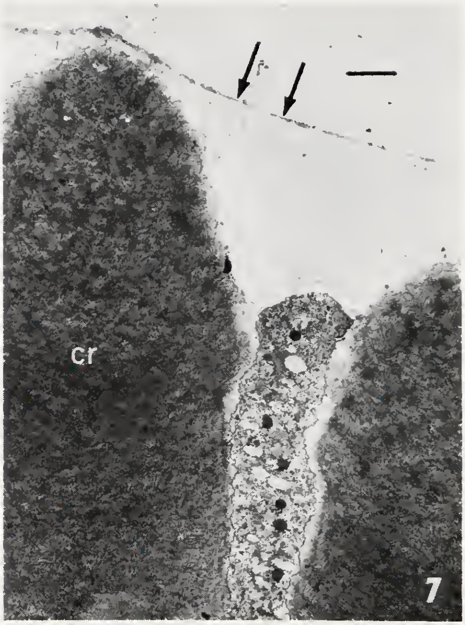
Using this approach, the crypts in any one oocyte account for a volume of 1.17×10^6 μm^3, or 12% of the oocyte's total volume, and a membrane surface area of 6.63×10^5 μm^2. Since the crypt membranes are continuous with the oolemma, the oocyte has an oolemma surface area of 8.35×10^5 μm^2, of which 79.5% is contained in the crypts.

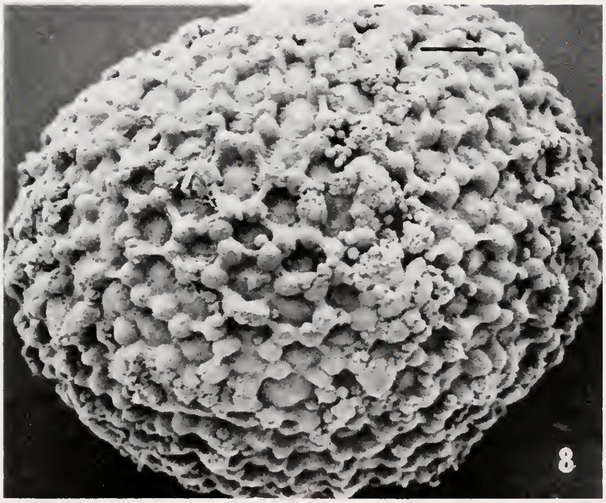
TABLE I

Egg and rod size

Unit	Sample size	Unactivated eggs	Activated eggs
Egg diameter	20	266 ± 13 μm	237 ± 10 μm
Rods per egg	10	746 ± 60	
Rod size			
Length	20	39.9 ± 1.5 μm	
Diameter			
Top	20	9.3 ± 2.3 μm	
Bottom	20	4.6 ± 1.3 μm	







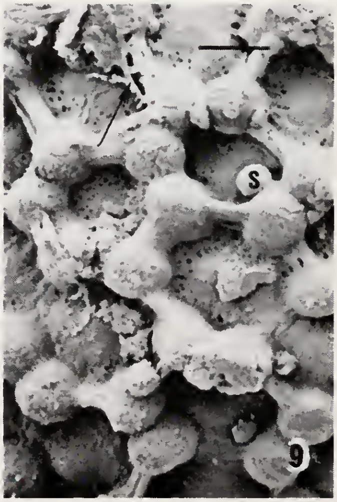


FIGURE 6. A light micrograph illustrating an oocyte at the beginning of its cortical reaction. The lifting of the investment coat (black arrow) and the appearance of a meiotic figure (white arrow) are shown. Bar = 50 μm.

FIGURE 6a. The first meiotic metaphase. Bar = 10 μm.

FIGURE 7. An electron micrograph of cortical rods emerging and lifting the investment coat off the egg. Bar = 1 μm.

FIGURE 8. A scanning electron micrograph showing early stages of the cortical reaction. Bar = 30 μm.

FIGURE 9. The emerging rods are clearly shown lifting the investment coat (arrows) and a few sperm(s) from the egg surface. Bar = 10 μm.

Early reaction

At this stage of the reaction, the rods slightly protrude from the surface of the oocyte (Figs. 6, 7), creating the blebbed appearance previously noted with phase microscopy. While there are no apparent changes in the substructure of the rods, their protrusion coincides with the lifting of the investment coat from the egg surface. When viewed with SEM, the emerging cortical rods appear as prominent blebs in the investment coat (Fig. 8). At a higher magnification, the investment coat is seen stretched between emerging rods and undergoing a partial loss of integrity (Fig. 9). Sperm still are seen closely associated with the egg surface (Fig. 9).

The first meiotic division becomes apparent during activation, as evidenced by metaphase figures in the peripheral cytoplasm of the oocytes (Figs. 6, 6a).

Corona stage

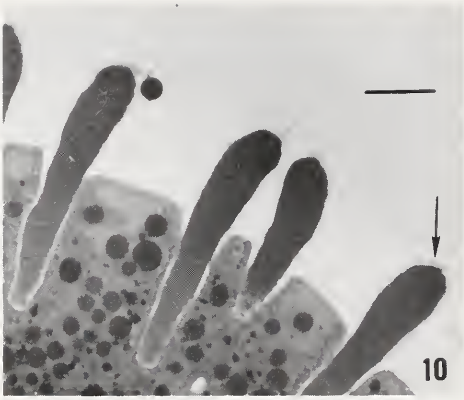
By this stage of activation, the cortical rods are well out of their crypts (Figs. 10, 11, 12). There are still no apparent changes in the substructure of the rods, though membranous vesicles are noted between the rod and crypt membranes as well as along the outside of the oolemma (Fig. 11). The integrity of the investment coat is lost almost entirely, and when viewed with the SEM, the coat appears only as fragmented strands stretched between cortical rods (Fig. 12.). Elimination of the investment coat reveals small depressions in the surface of the oolemma as well as numerous sperm still associated with the surface of the egg at this stage of development (Fig. 12). Transmission microscopy clearly shows these sperm, which apparently have lost their terminal spikes and undergone activation (Clark, Talbot, Neal, Mock and Salser, 1974), in alliance with the egg oolemma (Fig. 13). An electron-dense material always is noted along the periphery of the activated surface of the sperm (Fig. 13) as well as in association with the opposed gamete membranes (Fig. 14).

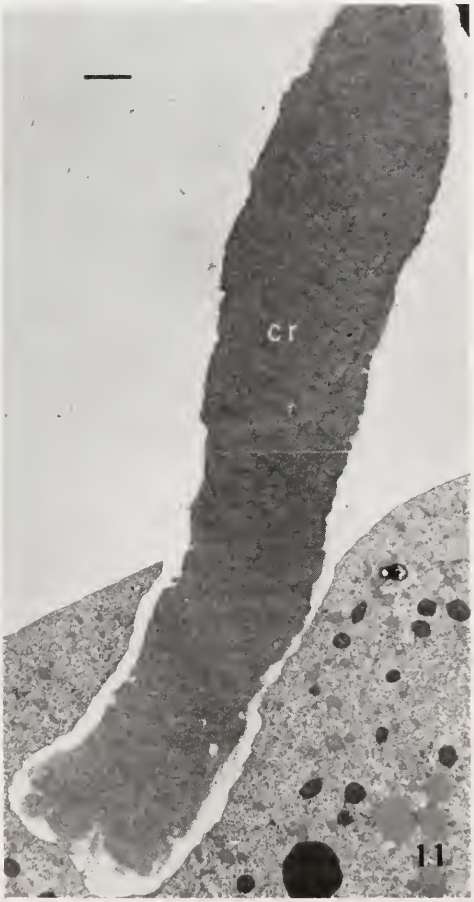
Dissipation stage

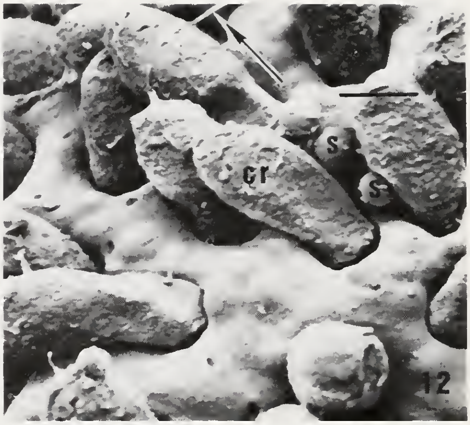
As the cortical rods are released from their crypts, they undergo notable changes. There is a distinct swelling at both their basal and apical ends accompanied by a loss of substructural integrity in these regions (Figs. 16, 17). The "bottle brush" structures appear to break down, resulting in a flocculent material that eventually surrounds the egg (Fig. 17). During this period of rod dissipation, membranous vesicles, as noted earlier, become more apparent (Figs. 17, 18, 19). These vesicular elements, which are initially associated with the crypts, become dispersed around the entire egg surface (Figs. 18, 19). Eggs, initially 266 μm in diameter, measure 237 μm in diameter once the cortical reaction is completed.

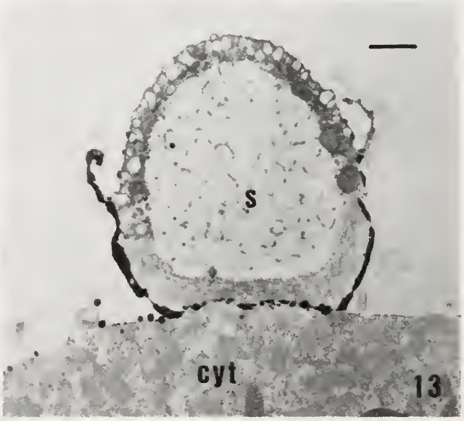
Discussion

The cortical reaction typical in the eggs of penaeid shrimp is unique with respect to the absence of membrane fusion during the reaction, the size of the cortical specializations, and the rapid expulsion and dissipation of these specializations in response to sea water. Few examples of such a massive cortical reaction are reported in the literature. A morphologically similar reaction occurs in the oocytes of *Nereis limbata* (Novikoff, 1939; Costello, 1949; Fallon and Austin, 1967). Fibrous whorls of large "alveolar vesicles" form a broad cytoplasmic band at the cortex of *Nereis* eggs. This alveolar material is released at fertilization and forms a jellylike layer around the egg that remains throughout early development of the zygote. The fibrous nature of the cortical alveolae is similar to the cortical rods; however, the alveolae lack the feathery substructure.









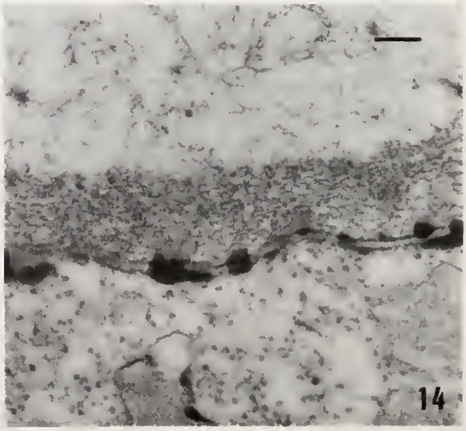


FIGURE 10. A thick plastic section of the corona stage. The arrows indicate the investment coat. Bar = 10 μm.

FIGURE 11. A transmission electron micrograph showing a cortical rod extending out of its crypt. Bar = 2 μm.

FIGURE 12. A scanning electron micrograph of an activated egg surface. Cortical rods (CR) are well out of their crypts. Occasional strands of investment coat extending between

Teleost eggs also undergo a massive cortical reaction (Yamamoto, 1939), retaining their extruded jellylike material beneath a vitelline membrane. This is in contrast to the penaeid eggs, which rapidly lose their dispersed cortical substances into the surrounding media.

In teleost eggs, the cortical reaction is initiated by sperm-egg contact and proceeds in a wavelike fashion from the point of sperm attachment (Yamamoto, 1939, 1961). The reaction in nereid eggs is also in direct response to sperm attachment (Costello, 1949), while in penaeid eggs, the reaction is stimulated by exposure to sea water and not by fertilization (Clark and Lynn, 1977). Although the cortical reaction in nereid eggs is initiated by sperm attachment, it is similar to the penaeid reaction in that it occurs simultaneously over the entire surface of the egg.

Another feature of penaeid ova is the decrease in the egg volume after the cortical reaction. A decrease in volume after the cortical reaction is also seen in the ova of the brook lamprey, *Entosphenus* sp. (Okkelberg, 1914), several echinoderms (Glaser, 1914; Eddy and Shapiro, 1976), and the anthozoan *Bunodosoma* sp. (Dewel and Clark, 1974). In *Bunodosoma* sp. eggs (Dewel and Clark, 1974), large regions of peripheral cytoplasm, interstitial to reacting and fusing cortical granules, are lost during egg activation (Dewel and Clark, 1974). Echinoderm eggs accommodate additional membrane from cortical vesicles by the elongation of surface microvilli (Eddy and Shapiro, 1976), while in penaeids, the excess membrane appears to be lost by a process of membrane vesiculation. The actual penaeid egg surface closely approximates the unactivated egg surface minus the crypt membrane and crypt apices surface area, though the volume lost during activation can be accounted for only partially, 50%, by initial rod volume. The mechanics of these reductions in cytoplasmic volume in penaeid eggs are poorly understood as are the functions of the cortical reaction itself.

Removal of supernumerary sperm from the surface of many animal ova is partially the result of a cortical reaction at the time of fertilization (Epel, 1975, for review). Many sperm on the surface of penaeid eggs are lifted off with the investment coat during the expulsion of the rods. Removal of supernumerary sperm from shrimp ova, however, does not appear to be the primary function of the cortical reaction, since the reaction is initiated by contact with sea water and not by sperm penetration. Two additional arguments against the cortical reaction's being a block to polyspermy are, first, the sperm are nonmotile and come into contact with the ova at the time of spawning (Clark *et al.*, 1974); and second, the large number of sperm attached to penaeid ova, as well as several sperm pronuclei within the oocytes (unpublished data), indicate that polyspermy is normal. This is not surprising since physiological polyspermy is known in many arthropods and is thought to be a prerequisite for successful fertilization (Austin, 1965, for review; Brown and Knouse, 1967; Hinsch, 1971).

Another possible function of the cortical reaction is the formation of a protective jelly layer. Such a layer may function as protection against an unstable environment (Allen, 1958; Schuel, 1978) as do the egg jelly or investment layers around

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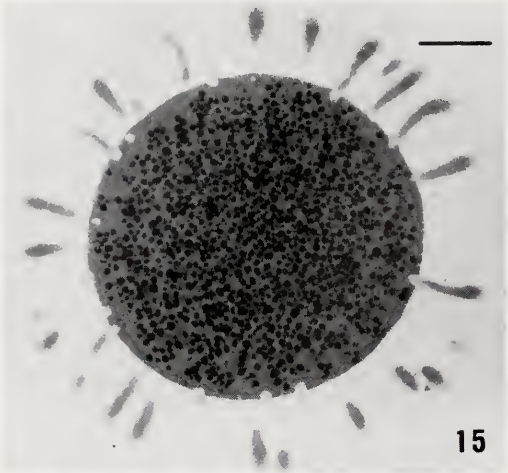
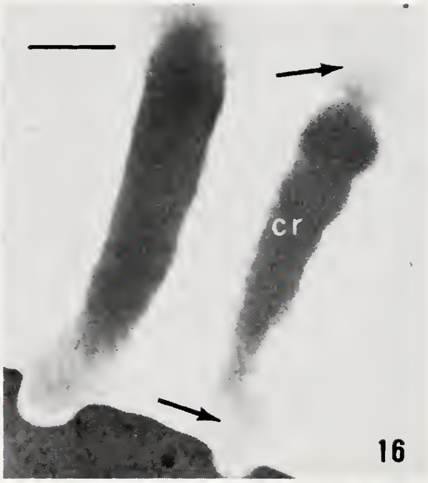
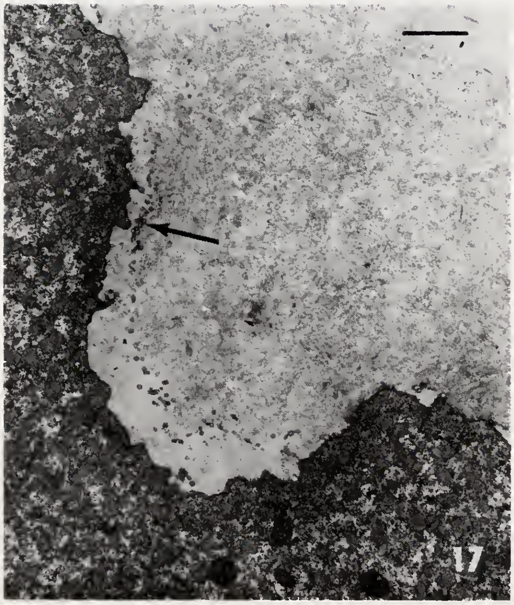
the rods (arrow) and sperm(s) are seen associated with the rods and oolemma. Small depressions are noted in the surface of the oolemma. Bar = 10 μm.

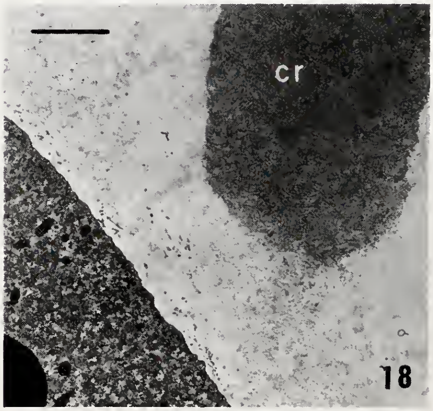
FIGURE 13. Transmission electron micrograph of sperm(s) in close association with an egg. Egg cytoplasm (CYT). Bar = 1 μm.

FIGURE 14. A higher magnification showing the membranous association between the sperm and egg. Bar = 0.2 μm.









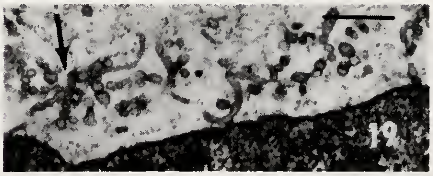


FIGURE 15. A light micrograph of an oocyte during the initial stages of cortical rod dissipation. Bar = 50 μm.

FIGURE 16. A higher magnification showing the cortical rods dissipating (arrows). Bar = 10 μm.

FIGURE 17. A transmission electron micrograph illustrating the base of a crypt, dissociated rod, and numerous small vesicular elements (arrow). Bar = 1 μm.

FIGURE 18. A fine structural view of cortical rod (cr) dissociation. Again, vesicular elements are noted along the oolemma. Bar = 2 μm.

FIGURE 19. A higher magnification of the membrane vesiculation (arrow) occurring directly adjacent to the oolemma. Bar = 0.2 μm.

many ova. Hudinaga (1942) reported that the dissipated cortical rod material is retained as a jelly coat until the second cleavage in *P. japonicus*. In ova of *P. aztecus* and *P. setiferus*, however, introduction of colloidal carbon to a slide of reacted eggs reveals no zone of jelly material after the formation of the hatching membrane. At present, several lines of investigation are in progress to determine the function of the cortical reaction. We also have initiated experiments to increase understanding of the process of fertilization and the effect, if any, of the cortical reaction on sperm-egg interaction.

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SUMMARY

The morphology of large cortical specializations (rods) located in the peripheral ooplasm is described in oocytes of the shrimp *Penaeus aztecus* and confirmed with *P. setiferus.* The rods, which are perpendicular to the oolemma, are "club"-shaped and approximately 39.9 μm long, with an apical diameter of 9.3 μm and a basal diameter of 4.6 μm. They are composed of numerous, tightly packed, fibrillar structures. Each cortical rod lies within a partially membrane-bound crypt and is separated from the external media by a thin investment coat. The investment coat lies directly adjacent to the oolemma and completely surrounds the egg. Upon exposure to sea water, the cortical rods begin to emerge from the crypts and appear to lift the investment coat off the egg surface. A corona is formed around the oocyte as the rods are expelled, but quickly dissipates. Extensive membrane vesiculation associated with cortical rod crypts is apparent at this time.

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RESPIRATION IN SOME ATLANTIC REEF CORALS IN RELATION TO VERTICAL DISTRIBUTION AND GROWTH FORM

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The rates of respiration of lower animals are determined, to a large extent, by a series of phenotypic adaptations or acclimations to specific factors in their environments. The integrated effects of these can be observed by comparing the rates of respiration of animals from habitats which are subject to different combinations of environmental factors (Davies, 1967). In hermatypic corals, those colonies living at greater depths on the reef experience both qualitative and quanitative differences in light regime. This in turn results in considerably lower rates of photosynthetic carbon fixation by the zooxanthellae in the coral tissues (Davies, 1977). Since it is now clear that a significant part of the fixed carbon is translocated to the coral tissues (Muscatine, 1973), it is possible that the overall nutritional input is lower in corals living at greater depths. Since respiration rate may be determined by the level of food intake (Davies, 1967), this study was undertaken to investigate possible intraspecific differences in the rates of respiration of corals, associated with habitat depth, and also to compare the rates of respiration of corals of different growth forms.

Although a number of earlier studies have presented data on the rates of respiration of corals (e.g. Yonge, Yonge and Nicholls, 1932; Kawaguti, 1937; Franzisket, 1969), comparisons of rates between different specimens of coral were not possible since the quantities of coral tissue involved were not measured. Ideally, respiration rates should be expressed on a unit basis which relates to the mass of respiring tissue, and which is not itself subject to change with the environmental factors being studied. In this paper, respiration rates are expressed in terms of the dry weight of the respiring coral tissue (including its contained zooxanthellae). Values are also given in terms of the surface area of the colony occupied by coral tissue since this is of particular value in studies on the primary productivity of corals. All experiments were carried out at the Discovery Bay Marine Laboratory, Jamaica.

MATERIALS AND METHODS

The following species were examined: *Montastrea annularis* (massive growth form) depth 3 to 40 m; *M. cavernosa* (massive), depth 13 to 80 m; *Acropora palmata* (branching), depth 5 m; *A. cervicornis* (branching), depth 15 m; *Agaricia lamarcki* (plate-like), depth 40 m; *A. undata* (plate-like), depth 65 m; *A. grahamae* (plate-like), depth 80 m. Coral heads of about 5 cm diameter were collected using SCUBA, with the exception of those from depths of 65 and 80 m which were collected by a NEKTON submersible (General Oceanographics Inc.). The corals were brought ashore in buckets of sea water and transferred to a running seawater aquarium without exposing the animals to the air. Any corals which were found to have an endozoic or burrowing fauna were rejected. Regrettably, all of the specimens of *Montastrea cavernosa* collected from 80 m were completely riddled with boring sponges, which invalidated any attempts at measuring their rates of respiration. The corals were kept overnight before use and inspected to ensure that the polyps expanded naturally and there was no apparent tissue damage.

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Rates of oxygen consumption were measured polarographically with a Beckman 221 oxygen electrode, inserted into a closed plexiglass chamber. The chamber, which had a volume of 253 ml, had a magnetic stirrer in the base and was surrounded by a water jacket maintained at 28° C. All measurements were carried out in darkness by covering the chamber with opaque black PVC sheet. The rates of respiration, which were not affected by the drop in oxygen partial pressure down to 50% of saturation, were derived from a linear pen-recorder trace. The duration of a single run was never more than 30 min.

At the end of the experiment, the volume of the coral was determined by measuring the volume of water in the chamber and subtracting from the volume of the chamber. The surface area occupied by living coral tissue was then measured by cutting and shaping aluminum foil to the coral head and then weighing the foil (Marsh, 1970; Johannes and Tepley, 1974). The head was then fixed in 10% formaldehyde for 24 hr before being decalcified in 10% HCl. (Future use of formaldehyde in this technique is not recommended because of the possibility of the formation of carcinogenic Bis-chloromethyl ether.) This procedure left an intact sheet of fixed coral tissue, from which the tufts of boring filamentous algae could be lifted. The tissue was then dried at 105° C for 24 hr.

In order to make an estimate of the loss of tissue material as a result of the fixation and acid treatment, a check was subsequently carried out using the British sea anemone *Actina equina*. Each anemone was divided into three pieces by means of radial cuts, and blotted to superficial dryness on filter paper. The first piece was weighed and then dried at 105° C, the second piece was weighed, immersed in 10% formalin for 24 hr and then dried and the third piece had an additional 24 hr in 10% HCl. Using six replicates it was found that the mean dry weight of the untreated piece was 19.0% of the fresh weight. The formalin treatment resulted in a mean loss of 9.2% of the dry weight and the formalin and acid treatment resulted in a further loss of 2.0%. It must therefore be borne in mind that the values for dry weights of coral tissue may be about 10% too low and that the unit-weight respiration rates would therefore be about 11% too high.

RESULTS

Intra- and interspecific variations with depth

Specimens of *Montastrea annularis* were examined from water depths of 2, 10, 20 and 40 m. From Figure 1 and Table I it can be seen that the rate of weight-specific oxygen consumption decreases progressively with increase in depth, so that the respiration of the deepest corals is less than half of that of the shallow-water forms. The greatest difference is observed in the relatively small depth change between the shallow-water colonies living at 2 m when compared with those living at 10 m.

Similarly, a change is noted when comparing specimens of *Montastrea cavernosa* from 13 m (the shallowest depth at which it occurred) with specimens living at

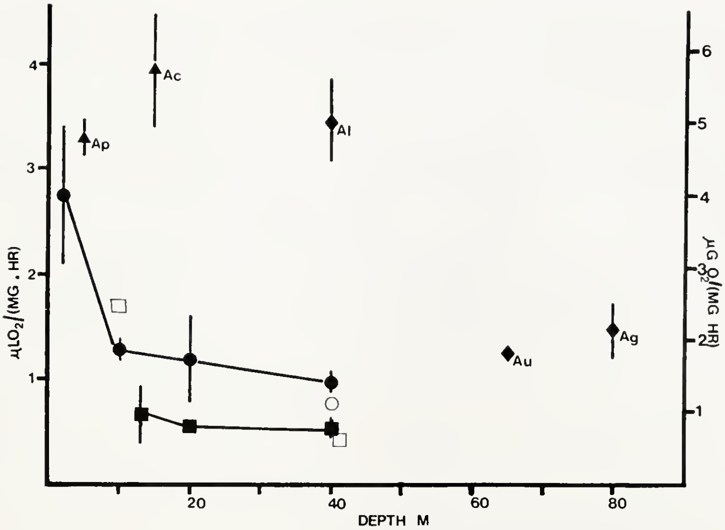

FIGURE 1. Weight-specific respiration rate of corals plotted against the depth at which the animals were living. Solid triangle, *Acropora:*—Ap *Acropora palmata;* Ac *Acropora cervicornis;* solid diamond, *Agaricia*—Al *Agaricia lamarcki;* Au *Agaricia undata;* Ag *Agaricia grahamae;* solid circle, *Montastrea annularis;* solid square, *Montastrea cavernosa.* The two open symbols represent specimens transplanted from shallow to deep water and *vice versa.* Vertical bars indicate standard deviations.

20 m and 40 m. The decrease in rate of respiration with increase in depth is also evident when surface-area-related respiration rate is plotted (Fig. 2).

Most other Caribbean corals exhibit a much more restricted depth-distribution range, rendering intraspecific comparisons impossible. However, it is possible to make interspecific comparisons among species of *Agaricia* with very similar growth forms, but with differing depth distributions. Again from Figures 1 and 2 it can be seen that specimens of *Agaricia lamarcki* from 40 m have markedly higher rates of respiration than those of *A. undata* from 65 m or of *A. grahamae* from 80 m.

In order to test whether the intraspecific differences in respiration rates are phenotypic responses to the particular environment at that depth, reciprocal transplantation experiments were carried out. Specimens of *M. cavernosa* and *M. annularis* from 40 m were transplanted to a depth of 10 m and *vice versa.* After 5 weeks they were removed from the reef, and their respiration rates measured. It was noted in both species that the 40-m specimens transplanted to 10 m had expelled most of their zooxanthellae but the corals nevertheless were apparently in good condition. The shallow corals transplanted to 40 m showed no apparent change. Regrettably, because of logistic difficulties, respiration-rate values are only available for one specimen from each transplantation site and the specimens of deep-water *M. annularis* transplanted to 10 m were lost. Figure 1 shows that in both species the rates of respiration had changed in the 5 weeks following transplantation: those transplanted to deep water had lowered their respiration rates, and *vice versa.* This suggests that in those species with a wide depth-distribution range, the depth-related respiration rate may be a phenotypic response to environmental factors.

TABLE 1

Respiration rates of corals, expressed on a unit basis of mg dry weight of tissue and surface area of living tissues ± standard deviations.

	n	Respiration rate			n	Respiration rate	
		µl O_2/(mg·hr)	µl O_2/(cm^2·hr)			µl O_2/(mg·hr)	µl O_2/(cm^2·hr)
Montastrea annularis				*Montastrea cavernosa*			
2 m	3	2.75±0.7	18.05±7.2	13 m	3	0.78±0.26	9.21±1.8
10 m	4	1.27±0.06	12.25±2.5	20 m	3	0.64±0.03	6.73±1.5
20 m	3	1.21±0.4	14.13±3.4	40 m	2	0.61±0.09	4.72±0.6
40 m	3	0.99±0.12	10.34±1.4				
Acropora palmata				*Agaricia lamarcki*			
5 m	2	3.32±0.13	18.8 ±2.3	40 m	2	3.49±0.6	8.95±1.9
Acropora cervicornis				*Agaricia undata*			
15 m	2	3.96±0.07	7.9 ±0.31	66 m	1	1.26	5.53
				Agaricia grahamae			
				80 m	3	1.52±1.2	3.2 ±0.7

Variations with shape

One of the most easily quantified measures of growth form is the surface area to volume (S/V) ratio, and Porter (1976) has used this ratio to demonstrate a correlation between polyp size, degree of heterotrophy, and growth form. From Figure 1 it is clear that at any depth considered, the branching forms (*Acropora palmata* and *A. cervicornis*) and plate-like forms (*Agaricia lamarcki*) have higher rates of respiration per unit weight of tissue than the massive *Montastrea* species.

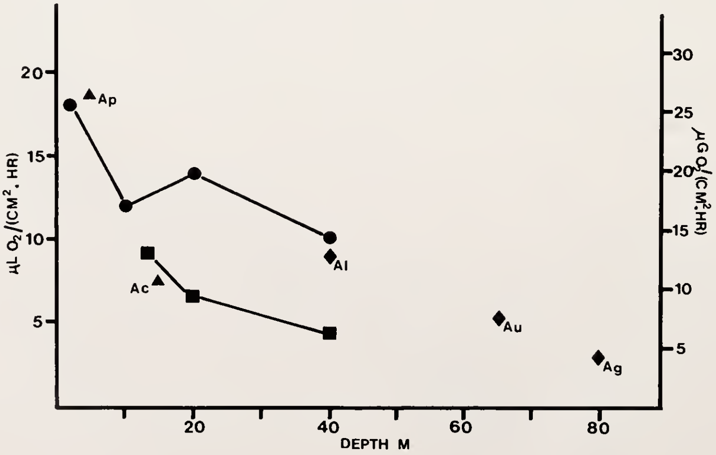

FIGURE 2. Surface-area-related respiration rate of corals plotted against the depth at which they were living. Symbols as in Figure 1

When the respiration rate is plotted against the S/V ratio for each coral (from Davies, unpublished) an almost linear relationship emerges (Fig. 3).

DISCUSSION

Most previous workers who have measured the rates of respiration of corals have used the values obtained to compare oxygen production in the light to respiration in darkness (*e.g.* Yonge *et al.*, 1932; Kanwisher and Wainwright, 1967; Roffman, 1968), in which case a unit-weight or surface-area basis of comparison is not necessary. Kawaguti (1937) and Pillai and Nair (1972) did express results on a unit-weight basis but used the weight of the whole colony. This has the disadvantage of underestimating the rate of respiration by the living tissue, particularly in massive corals where the weight of skeleton may be 30 times that of the tissue. Recently McCloskey, Wethey and Porter (1978) have drawn attention to the need to adopt a standard unit for expressing both respiration and photosynthesis by corals, and have recommended the use of the unit of grams nitrogen of coral tissue. In the present study, results have been expressed in terms of both surface area and dry weight of coral tissue. Except in highly branched corals, surface area is simple to measure; and in studies on productivity, where knowledge of the area available to gather incident light is of prime importance, this method is probably preferable. However, the biomass of tissue per unit of surface area varies among coral species. Thus in Figure 2 it would appear that *Acropora palmata* has a higher rate of respiration than *A. cervicornis*. However, *A. palmata* has a very much greater weight of respiratory tissue per unit surface area than *A. cervicornis* (Davies, unpublished). When compared on a unit-weight basis (Fig. 1) it is apparent that *A. cervicornis* has the higher rate of respiration. For investigation of energy budgeting in corals or for comparison of energy expenditure under different conditions, unit-weight-related oxygen-consumption measurements are to be preferred.

In both *M. annularis* and *M. cavernosa* there is a decrease in weight-specific respiration rates with increase in water depth. The same observation is apparent when making interspecific comparisons between *Agaricia lamarcki* and the deeper

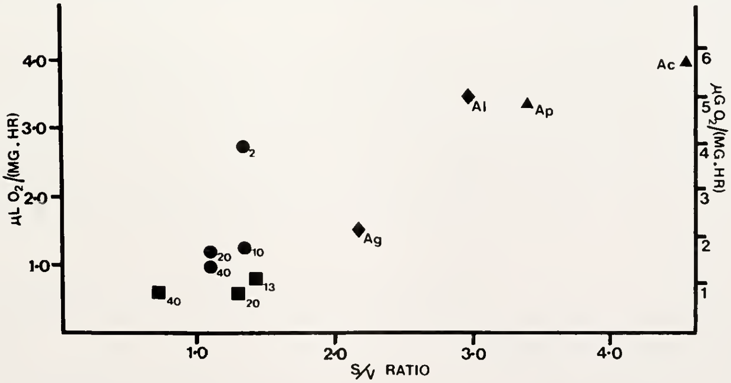

FIGURE 3. Weight-specific respiration rate of corals plotted against surface area to volume (S/V) ratio. Solid triangle, *Acropora*—Ap *Acropora palmata;* Ac *Acropora cervicornia;* solid diamond, *Agaricia*—Al *Agaricia lamarcki;* Ag *Agaricia grahamae;* solid circle, *Montastrea annularis;* solid square, *Montastrea cavernosa.* The depths (m) at which the *Montastrea* specimens were living is indicated.

water *A. undata* and *A. grahamae*. Since a proportion of the oxygen consumption can be attributed to the zooxanthellae, which may have different rates of respiration per unit weight than the coral tissue, the depth-related differences in respiration rates of the colonies might be explained by changes in the numbers of zooxanthellae in the tissues. However, Drew (1972) demonstrated in a series of Indo-Pacific corals that there was almost no change in the numbers of zooxanthellae per cm^2 of coral tissue with increasing depth, and preliminary experiments carried out in the present study confirmed this.

It is likely, therefore, that the respiration differences represent true differences in energy expenditure. If it can be assumed that the energy requirements for maintenance (i.e. for steady-state active transport and biosynthesis) of a unit weight of tissue would be the same in a shallow-water as in a deep-water coral, then the difference could be accounted for by the energy expenditure in biosynthesis for growth. Evidence for decrease in growth rate with depth in *Meandrina meandrites* has been obtained by Bak (1976) who measured skeletal weight underwater, and by Barnes and Taylor (1973), who demonstrated that the rate of uptake of ^{45}Ca was 55% higher in the central corallites of shallow-water (9 m) specimens of *Montastrea annularis* when compared with those living at 33 m. However, it should be borne in mind that in the present series of experiments the rate of respiration was measured in darkness, when skeletogenesis would be at a diminished rate (Pearse and Muscatine, 1971; Chalker and Taylor, 1975). Nevertheless, it is possible that for the duration of the experiments tissue growth was continuing. On the assumption that corals with a high rate of skeletal growth have a correspondingly high rate of tissue growth, it is likely, therefore, that we are here observing depth-related differences in energy consumption for the growth of new tissues.

A hitherto unsuspected correlation of respiration rate with growth form is suggested by Figure 3. The higher the S/V ratio, the higher is the rate of respiration per unit weight of tissue. Again, it is probable that this can be explained on the basis of differences in growth rates, since Bak (1976) has shown that the growth rate, or increment of new skeleton added per unit time, is higher by a factor of about 10 in high S/V forms (*Acropora palmata, Agaricia agaricites*) when compared with corals, such as *Montastrea annularis* and *Meandrina meandrites*, which have a low S/V ratio.

The actual rate of growth of any coral will be determined by both intrinsic factors, such as growth form, and by environmental factors, which include the level of nutritional intake. Porter (1976) has argued from morphometric grounds that high S/V corals, with small polyps, are adapted to a metabolic economy based largely upon the nutritional input of carbon compounds synthesised by the zooxanthellae. Their high respiration rates and growth rates are indications of the success of this.

In comparing deep-water and shallow-water corals of the same growth form, the lower respiration rates and growth rates of the former may be attributable to a low level of nutritional input.

It has been shown (Davies, 1977) that the quantity of carbon fixed by zooxanthellae in deep-water specimens of *Montastrea* may be only 20–25% that of shallow water specimens. If the intake of zooplankton is similar at the two depths, then the total nutritional input will be lower in the deep-living corals. In this way, respiration rate and growth rate would be determined primarily by the change in input of carbon compounds by the zooxanthellae with change in light intensity accompanying change in depth.

I wish to acknowledge grants from the Royal Society, the Carnegie Trust, and the University of Glasgow; the assistance of colleagues at the Discovery Bay Marine Laboratory, and Dr. L. S. Land for the use of the NEKTON submersible for collection of the deep-water corals.

SUMMARY

1. The rate of oxygen consumption of a number of Caribbean corals was determined, in darkness, and the results expressed on a unit-weight and unit-surface-area basis to enable inter- and intraspecific comparisons to be made.

2. Intraspecific comparisons of *Montastrea annularis* and *M. cavernosa* and comparisons between species of *Agaricia* show that corals living in deep water (40 m) have lower rates of respiration than shallow-water corals.

3. Corals, such as *Acropora* and *Agaricia*, with a high surface-to-volume ratio have higher rates of respiration per unit weight than the massive corals like *Montastrea.*

4. It is suggested that differences in rate of respiration are the result of differences in energy expenditure in the biosynthesis of tissue growth and that this is determined to a large extent by the nutritional input into the colony from the zooxanthellae.

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THE MORPHOLOGY OF *HYDROBIA TRUNCATA* (GASTROPODA: HYDROBIIDAE): RELEVANCE TO SYSTEMATICS OF *HYDROBIA*

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The genus *Hydrobia* is of considerable current interest to ecologists and systematists. Hydrobiids comprise one of the most important groups of deposit-feeding invertebrates to be found in estuaries and salt marshes of eastern North America and northern Europe. For example, an exceptionally high density of 42,000 individuals/m^2 may be attained in the mid-intertidal offshore bank of an estuary (Russell-Hunter and Russell-Hunter, 1962). Considerable attention has been given to how various hydrobiid species are deployed, use resources, interact when sympatric, and occupy different niches (Russell-Hunter and Russell-Hunter, 1962; Newell, 1965; Fenchel, 1975; Hylleberg, 1975; Fenchel, Kofoed, and Lappalainen, 1975; Fenchel and Kofoed, 1976; Levinton and Lopez, 1977; Lopez and Levinton, 1978; Wells, 1978).

While the species status within northern European *Hydrobia* has seemingly been clarified (Muus, 1967), this is not the case in North America. There is no systematic study comparing species of *Hydrobia* along the east coast of North America. *Hydrobia totteni* Morrison (1954) and *Hydrobia salsa* Pilsbry (1905) from New England have been characterized by salinity preferences, diameter of egg capsules, and diameter of eggs (Mazurkiewicz, 1972). The external topography, radula and male reproductive system of *Hydrobia totteni* have been studied (Davis, 1966). However, because *Hydrobia truncata* Vanatta (1924) has not been studied, because the detailed comparative anatomy of all organ systems of *H. salsa, H. totteni,* and *H. truncata* has not been presented, and because it is suspected that there is a complex of undescribed brackish-water hydrobiid species along the east coast of North America (Morrison, 1970), we cannot be sure which species has really been studied in current ecological literature.

The purposes of this paper are to present a detailed morphological study of *Hydrobia truncata* Vanatta to be used for comparison within and between populations of North American *Hydrobia,* and to show that three species of *Hydrobia* live in the same geographic area in New England. Detailed morphology is presented for four reasons: It has been shown that clarification of the systematic relationships among hydrobioid snails (*i.e.* those resembling hydrobiids in shell, penis, radula, and operculum) is dependent on morphological studies that include all organ systems, and that characters seen in the female reproductive system, entire male reproductive system, nervous system, and radular teeth have thus far best served to delineate taxa of hydrobioids (Davis, 1979). The Hydrobiidae of authors pre-1979 were a worldwide family consisting of about 100 genera and over 1000 species. However, it has been shown by detailed morphological studies that the Hydrobiidae *sensu lato* are polyphyletic (Davis, 1979). The large North American hydrobioid fauna of some 25 genera and 100 species, mainly of fresh water (Thompson, 1968), is poorly understood. There are no detailed morphological studies on North American *Hydrobia* or other related brackish water hydrobioid snails. The relationships between North American and northern European *Hydrobia* are unclear (Muus, 1963; Davis, 1966).

Ecological data are given for specimens of *Hydrobia truncata* from Stone Harbor, New Jersey, to facilitate comparisons with other taxa. A conchological comparison is made between paratypes of *H. truncata, H. salsa,* and other taxa referred to *H. totteni* and *H. truncata.*

MATERIALS AND METHODS

Localities

Specimens of *Hydrobia truncata* were collected from a salt water marsh at the Wetlands Institute, Stone Harbor, Cape May Co., New Jersey, from May to August, 1977–1978. They came from a shallow high intertidal pool in the marsh near a channel that emptied into the inlet at Stone Harbor. Other pools were present in the marsh; they appeared isolated but were connected during extremely high tides (E. Hoagland, personal communication). Salinity at high tide was determined using an American Optic salinometer. Snails were collected with a hand sieve from the substrate and algae.

Field excursions were made to the type locality at marshes of the Little Choptank River, Towne Point, Delaware-Maryland Peninsula, Dorchester Co., Maryland. *H. truncata* was not found there.

Anatomy

Dissections were carried out on more than 100 individuals at magnifications of 25× and 50× using a WILD M-5 microscope. Established techniques were used (Davis and Carney, 1973; and Davis, 1979). Specimens were prepared for histology by fixation in Bouin's solution and were sectioned at 7 μm and stained in hematoxylin and eosin. Shells were cleansed of periostracum and debris by soaking in Clorox (0.5% sodium hypochlorite). Radulae were obtained by dissecting out buccal masses and soaking them in Clorox to remove the soft tissue. After initial measurements and row counts, the radulae were permanently mounted in Euparol for study under high magnification, including oil immersion. Drawings of organs were prepared from sketches and checked against measurements for accuracy using the technique of Davis and Carney (1973).

Shells of the following populations were compared: *Hydrobia truncata* (holotype and paratypes, A.N.S.P. [Academy of Natural Sciences of Philadelphia] 132886); Stone Harbor, Cape May Co., New Jersey (A.N.S.P. 349361); Salt Pond, behind Falmouth Beach, Barnstable Co., Massachusetts, July, 1978, (A.N.S.P. 349374). *Hydrobia totteni,* Wild Harbor River, N. Falmouth, Buzzards Bay, Barnstable Co., Massachusetts, September, 1969 (A.N.S.P. 349375); Provincetown, Barnstable Co., Massachusetts, 1884 (A.N.S.P. 134452); Katama Bay, Martha's Vineyard, Dukes Co., Massachusetts, 1875 (A.N.S.P. 102648). The type of *H. totteni* has not been located and is presumed lost. *Hydrobia salsa* (holotype and paratypes, A.N.S.P. 90445) Cohasset, Norfolk Co., Massachusetts. Note that *Hydrobia minuta* (= *Turbo minuta* Totten, 1834) is a synonym of *H. totteni* (see Morrison, 1954).

TABLE I

Comparison between shell parameters of populations of Hydrobia *studied.* $\bar{X}$, *mean; s.d., standard deviation.*

Population	Parameters									
	Length last 3 whorls		Shell width/ Length last 3 whorls		Length body whorl/ Length last 3 whorls		Length aperture/ Width aperture		Length aperture/ Length last whorl	
	$\bar{X}$	s.d.	$\bar{X}$	s.d.	$\bar{X}$	s.d.	$\bar{X}$	s.d.	$\bar{X}$	s.d.
H. totteni (N = 25) Provincetown	2.56	0.28	0.61	0.02	0.74	0.03	1.47	0.09	0.47	0.03
H. totteni (N = 25) Martha's Vineyard	2.52	0.18	0.63	0.02	0.75	0.02	1.51	0.11	0.78	0.02
H. totteni (?) (N = 4) Wild Harbor River (sample II-1-Grassle)	2.56	0.28	0.56	0.01	0.75	0.03	1.52	0.06	0.47	0.03
H. salsa (N = 5) paratypes	3.18	0.10	0.64	0.01	0.76	0.02	1.48	0.06	0.49	0.04
H. truncata (N = 10) (Salt Pond, 7/78)	3.32	0.22	0.60	0.02	0.68	0.03	1.44	0.08	0.43	0.02
H. truncata (N = 25) Stone Harbor	2.81	0.20	0.58	0.03	0.69	0.02	1.48	0.08	0.44	0.02
H. truncata (N = 25) paratypes	2.59	0.14	0.56	0.02	0.67	0.02	1.54	0.05	0.43	0.02

Shells were considered to house mature animals if the parietal callus was complete. Length of the body whorl, width of the fourth whorl (counting adapically from the aperture), width of the body whorl, and length and width of aperture were measured on mature specimens. Because the shells of many populations have eroded apices or are decollate, one cannot compare sizes of individuals by total shell length. Accordingly, length of the last three whorls and length of the body whorl were used as indices of size (Davis, 1969a). Populations were compared using length of body whorl/length of the last three whorls; they were compared by width of the fourth whorl/length of the last three whorls. Computer-mediated comparisons of length of body whorl per length of the last three whorls were made for six populations. The statistical analysis system was used with the General Linear Model procedure for linear regressions. Regressions were compared using the standard *t* test. Other ratios were also employed (Table I).

RESULTS

Ecology

H. truncata was found only in the one pool at Stone Harbor, New Jersey that received regular tidal flow. The salinity was 32‰. The pool had a hard-packed mud-silt bottom paved with live and dead specimens of *Gemma gemma* (Totten) (Bivalvia); the pool was anoxic at the bottom. Specimens of *H. truncata* were found on the few bare patches, on the *Gemma* shells, and on immersed algae (red and green). The snails apparently preferred the green algae to the red. The snails were moderately abundant, as a single scoop of a hand sieve yielded 25 to 75 individuals. *Ilyanassa obsoleta* (Say), young specimens of *Mytilus edulis* Linné, *Mya arenaria* Linné, and an occasional specimen of *Sayella fusca* (C. B. Adams) (pyramidellid) were other molluscs found in the pool.

At low tide the depth of the pool was reduced to 7 to 15 mm; some areas of bottom were uncovered, leaving temporarily unconnected pools scattered about. The snails appeared to bury themselves just beneath the surface at low tide. At no time during the seven visits to the marsh at low tide was the entire bottom of the pool exposed.

Anatomy

External features. The head-foot morphology and pigmentation patterns of *H. truncata* are very similar to those described for *H. totteni* (Davis, 1966). The snout (Fig. 1A) is 0.58 mm long and darkly pigmented along most of its length with the anterior end (0.05-mm strip) unpigmented. The buccal mass is readily seen through the snout. The snout tip, when viewed anteriorly, is seen to have yellow granules imbedded within it (see Davis, 1966, Fig. 4). The cylindrical tentacles measure 0.85 mm from the eye lobes and extend beyond the tip of the snout. The tentacle tips are rounded and fringed with long cilia. Occasionally, snails have tentacles either bent along their length or forked near the tips. There are 5 to 8 ciliated tufts (hypertrophied) along the outer basal margin of the left tentacle as in *H. totteni* (Davis, 1966) (Fig. 1A). A black pigment bar, about 0.14-mm long, is medially placed near the tentacle tips. As in many hydrobioids, there are dense concentrations of yellow granules about the eyes (Fig. 1A). The concentration of granules in *Hydrobia* does not reach the extent of concentration in the Pomatiopsidae, where the lunate patch of glands

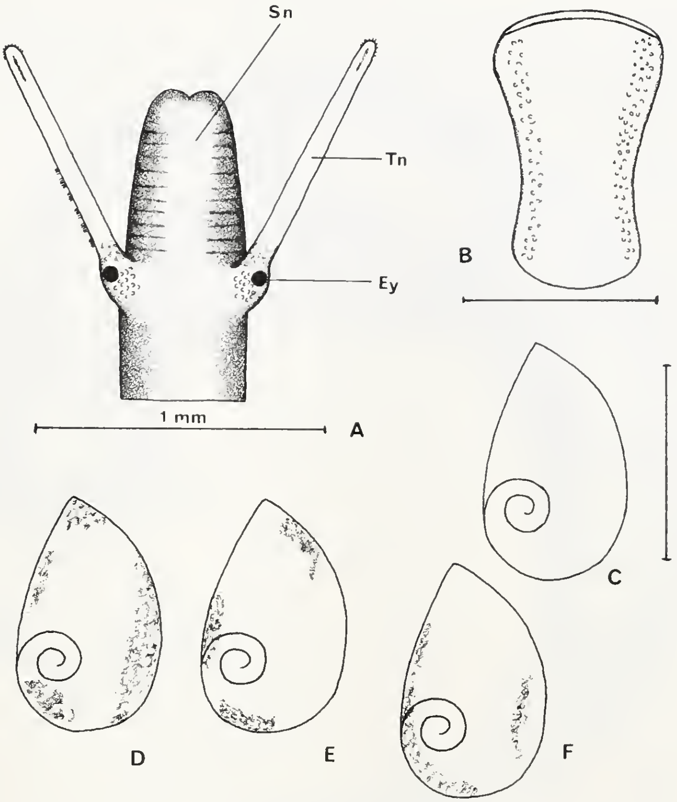

FIGURE 1. The head, foot, and operculum of Stone Harbor specimens of *Hydrobia truncata.* A: Dorsal view of the head; B: Ventral aspect of the foot showing anterior mucous slit; C: Operculum showing paucispiral condition. D–F: Variation in pigment pattern seen on the operculigerous lobe through the operculum.

is so dense that it has been called an "eyebrow." Similar granules are sparse within the tentacles. The eye lobes are only slight swellings compared to the bulging eye lobes of many pomatiopsid snails (Davis, 1967, 1979). Behind the snout the head and mantle cavity floor are peppered with white granules (Davis, 1966) except for a narrow strip along the center.

As in *H. totteni,* there is an unpigmented strip running obliquely along the side of the head-foot (Davis, 1966, Fig. 3). There is neither an omniphoric groove nor a pedal crease (contrast with *Pomatiopsis,* Davis, 1967). The foot (Fig. 1B), measures (with animal gliding) 1.4 mm by 0.88 mm, is elliptical in shape and is widest anteriorly. The sole is dotted along the sides with yellow granules. A narrow mucous slit is present along the anterior margin. The snails are agile and move rapidly by ciliary motion. Floating individuals are frequently found in the laboratory cultures.

A prominent yet small, highly ciliated pallial tubercle is seen emerging from the extereme right mantle edge at a point where the mantle folds dorsally to encircle the head (Fig. 2A, B). This fold in the mantle is directly opposite the path of the exhalent current along the neck of the animal, and corresponds to the most adapical end of the shell aperture when the snail emerges. This is the first

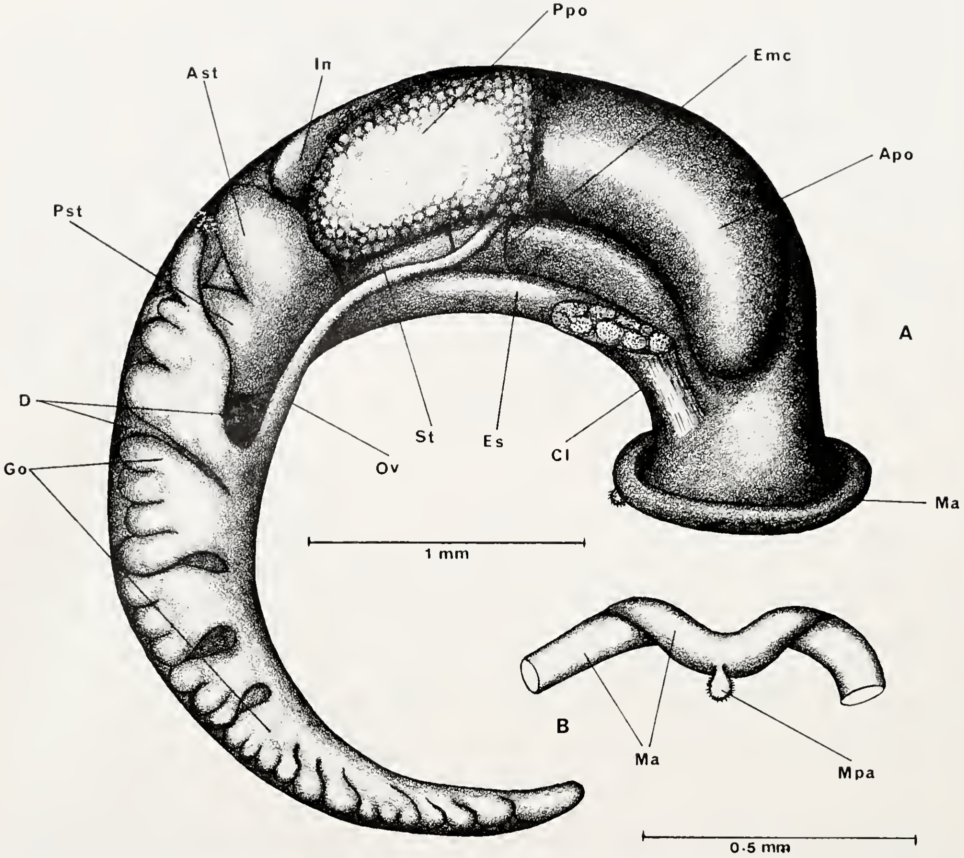

FIGURE 2. Female without head, showing organs as seen through the epithelium, but with kidney tissue removed to reveal the style sac (St), A; section of the mantle collar turned back to reveal the ciliated mantle papilla, B. Abbreviations are: Apo, anterior section of pallial oviduct (= capsule gland); Ast, anterior chamber of stomach: Cl, columellar muscle; D, digestive gland; Emc, posterior end of the mantle cavity; Es, esophagus; Go, gonad; In, intestine; Ma, mantle collar; Mpa, papilla of the mantle; Ov, oviduct; Ppo, posterior section of pallial oviduct (= albumen gland); Pst, posterior chamber of stomach; St, style sac.

description of such a tubercle in North American *Hydrobia*. *Hydrobia ulvae* of Europe has a much larger pallial tentacle, similarly located (Fretter and Graham, 1962, p. 581; personal observation; and Taylor, 1966), but no one has speculated as to its function. It may function as a sensory aid and/or assist in voiding excrement. It is found in both sexes. Body pigmentation consists of a dark dusting of melanin pigment on the dorsal surface with a much lighter dusting of pigment on parts of the ventral surface.

Operculum. The operculum (Fig. 1C) is corneous, thin and paucispiral with 2.5 to 2.75 rapidly expanding whorls. It is light brown in color. Its position on the operculigerous lobe is similar to that of *H. totteni* (Davis, 1966, Fig. 3). The pigmentation of the foot under the operculum varies from uniformly dark all around the perimeter to discrete patches along the perimeter (Fig. 1D–F). It does not differ much from that of *H. totteni* (Davis, 1966, Fig. 4).

TABLE II

Dimensions (mm) of non-neural organs and structures of Hydrobia truncata.

Organ or structure		No.	$\bar{X}$	s.d.	Range
Body					
(m)	L	5	5.34	0.134	5.16 – 5.52
(f)	L	7	6.26	0.446	5.80 – 7.06
Gill number					
(m)		8	21.12	1.36	19–23
(f)		6	23.3	2.25	19–25
Osphradium (m)	L	6	0.266	0.02	0.24 – 0.30
	W	6	0.083	0.008	0.08 – 0.1
Gonad (m)	L	7	2.990	0.226	2.68 – 3.22
	W	5	0.448	0.052	0.36 – 0.50
(f)	L	13	3.060	0.339	2.6 – 3.8
	W	10	0.628	0.042	0.56 – 0.68
Prostate	L	13	1.200	0.104	1.04 – 1.34
	W	13	0.417	0.056	0.26 – 0.48
Penis	L	9	1.530	0.210	1.27 – 1.94
	W	9	0.327	0.043	0.249– 0.388
Pallial oviduct	L	13	2.480	0.229	2.22 – 2.94
	W	11	0.589	0.056	0.50 – 0.70
Bursa copulatrix	L	14	0.366	0.046	0.3 – 0.42
	W	14	0.196	0.027	0.14 – 0.24
Seminal receptacle (body)	L	8	0.185	0.018	0.16 – 0.20
	W	8	0.121	0.008	0.11 – 0.14
Seminal receptacle (duct)	L	8	0.035	0.007	0.02 – 0.04
	W	8	0.048	0.010	0.04 – 0.06
Digestive gland (f)	L	7	2.760	0.337	2.3 – 3.3
Buccal mass (m + f)	L	14	0.704	0.118	0.56 – 0.98

Mantle Cavity. The organization of the mantle cavity and structure of the gills and osphradium are typically hydrobioid (see Davis, 1967; Davis, Kitikoon, and Temcharoen, 1976, for figures). Gill and osphradium statistics are given in Table II. The mantle cavity is about 1.7 mm long, all the length of which (except the anteriormost 0.18 mm) is occupied by the ctenidium. The osphradium is centered slightly less than half way back along the ctenidium.

Digestive System. The digestive system is typically hydrobioid. Two elongated salivary glands (length of 0.34 mm) open into the posterior dorsal buccal mass (Davis, 1969b, Fig. 32). As in other hydrobioids (Davis *et al.*, 1976), the salivary glands lie on top of the nerve ring and do not pass through the ring. The oesophagus enters the stomach between the two chambers (Figs. 2A, 4B). A single opening to the digestive gland is found. In both sexes the digestive gland extends to the tip of the snail and lies lateral to the gonad. No caecal appendix to the stomach (figured for *Pyrgula*, Radoman, 1955) was found in dissection or in section. Radoman (1973) has considered this structure to be a characteristic of the Hydrobiidae. The intestine exits anteroventrally from the style sac and bends back upon itself before running forward (Figs. 2A, 3A, 4B). Fecal pellets

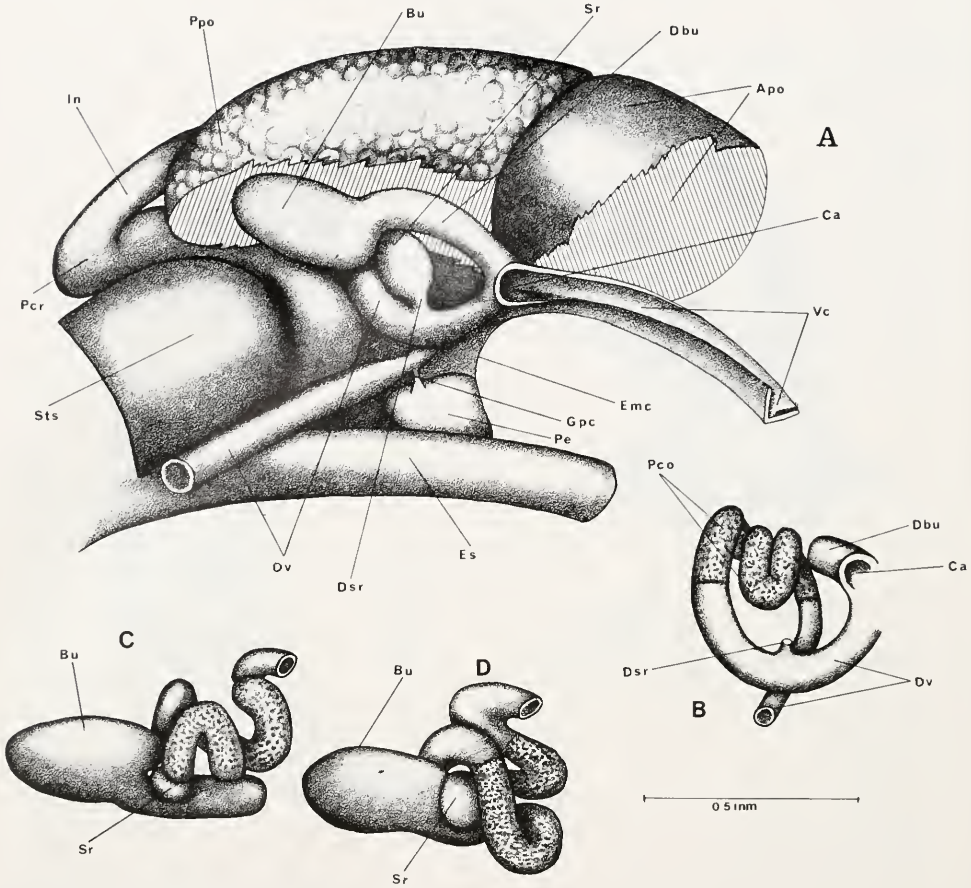

FIGURE 3. Female reproductive system. A: Same view as in Figure 2A, but restricted to the area immediately surrounding the bursa copulatrix complex. The epithelium and kidney tissue were removed. Much of the ventral section of the albumen gland (Ppo) was scraped away to reveal the bursa copulatrix (Bu) and seminal receptacle (Sr). Most of the capsule gland (Apo) was removed to reveal the interrelationship of the ciliated ventral channel (Vc) of the pallial oviduct, to the common aperture (Ca), to the duct of the bursa (Dbu), and the oviduct. The gonopericardial duct (Gpc) is clearly seen in this orientation, connecting the oviduct (Ov) to the pericardium (Pe). B; Same view as in Figure 2A, but with the bursa copulatrix and seminal receptacle removed to reveal the nature of the pigmented oviduct coils that are dorsal to the bursa in Figure 2A. C, D: The bursa copulatrix (Bu) seen in Figure 2A has been lifted and rotated to reveal how the seminal receptacle (Sr) tucks into the space caused by the juncture of the duct of the bursa and the projecting anterior end of the bursa. Abbreviations are: Apo, anterior pallial oviduct (= capsule gland); Bu, bursa copulatrix; Ca, common aperture of ventral channel, duct of the bursa, and oviduct; Dbu, duct of the bursa; Dsr, duct of the seminal receptacle; Emc, posterior end of the mantle cavity; Es, esophagus; Gpc, gonopericardial canal; In, intestine; Ov, oviduct; Pe, pericardium; Pco, pigmented coils of the oviduct; Pcr, pellet compressor; Ppo, posterior pallial oviduct (= albumen gland); Sr, seminal receptacle; Sts, style sac; Vc, ventral channel of the pallial oviduct.

are fusiform in shape and light brown in color, typical for hydrobioids (Taylor 1966).

The radula is typically taenioglossate. The tooth and cusp morphologies are as shown for *H. totteni* (Davis, 1966, Fig. 6). The central tooth has a single basal cusp originating from a pronounced lateral angle as is typical for the Hydrobiidae (Davis, 1966, 1979). As in *H. totteni,* there is a pronounced basal process on the central tooth. Radular statistics based on 12 individuals with mean and standard deviation are as follows: length, 0.569 ± 0.05 mm; width, 0.117 ± 0.01 mm; width of base of central tooth, 0.032 ± 0.003 mm; number of rows of teeth, 41 ± 1.86; number of rows in formative stage, 2.92 ± 1.08.

The various cusp arrangements on the four types of taenioglossate teeth are given with the percentage of radulae showing the formula at least once (in parentheses): central tooth; 3–1–3/1–1 (82), 4–1–4/1–1 (41), 4–1–3/1–1 (35), 3–1–2/1–1 (24), 5–1–4/1–1 (11): lateral tooth; 3–1–3 (86), 4–1–3 (79), 3–1–2 (43), 6–1–4 (7): inner marginal; 11 (77), 10 (53), 12 (47), 13 (18), 8 (12): outer marginal; 11 (88), 12 (62), 10 (56), 13 (25), 9 (25).

The formulae for the most common cusp arrangements in *Hydrobia truncata* are given with the percentage of radulae with this formula (in parentheses): central tooth, 3–1–3/1–1 (82); lateral tooth, 3–1–3 (86); inner marginal, 10–12 (94); outer marginal, 11–12 (100). The tooth morphologies, radula statistics and formulae vary little from those of *H. totteni* (Davis, 1966).

Female reproductive system. The female reproductive system is discussed in some detail because investigators of previous studies of North American hydrobiid anatomy have not discussed it (Davis, 1966) or have treated it incompletely (Thompson, 1974), and because this organ system has been useful in distinguishing clades within the hydrobioid grade of organization (Davis, 1968; Davis *et al.,* 1976; Davis, 1979).

The general organization of the female reproductive system is shown in Figures 2 and 3; organ measurements are presented in Table II. The ovary, which appears yellow-white in life, is long relative to body length (48.8%). There are usually 5 large anterior gonad branches and a variable number of small lobes posterior to them. The gonad is unusual in that the anterior-most gonadal lobe extends over the posterior stomach lobe, and the gonad always extends to the posterior tip of the digestive gland (Fig. 2A). Usually, hydrobioid ovaries occupy a fraction of the length of the digestive gland between the end of the stomach and the posterior tip of the snail (*e.g., Pomatiopsis,* Davis, 1967). The gonadal lobes have a swollen appearance in mature females and oocytes are frequently seen.

The oviduct, ventrally viewed, passes over the oesophagus-stomach juncture and disappears beneath the pallial oviduct (Fig. 2A). The pallial oviduct (Ppo and Apo) is quite large, averaging 2.49 mm in length. The two sections of this organ, the glandular posterior albumen gland (Ppo) and the anterior capsule gland (Apo), are readily distinguishable when the living animal is lightly stained with neutral red. The capsule gland appears smooth and firm while the albumen gland has distinct glandular pustules and appears flimsy. The capsule gland is the longer of the two sections of the pallial oviduct. The bursa copulatrix is found to be imbedded in the mid to posterior ventral part of the albumen gland. The bursa is revealed by gently scraping away the ventral and ventro-lateral parts of the albumen gland (Fig. 3A). The posterior tip of the bursa copulatrix is located about 0.24 mm anterior to the posterior end of the pallial oviduct; the ventral bursa is free of the albumen gland, covered with kidney tissue, and presses against the ventral surface of the oviduct. A single tube, the duct of the bursa, extends from the anterior end of the bursa (Dbu, Fig. 3A).

The pear-shaped seminal receptacle (Sr) is found snugly fitted between the anterior end of the bursa and the duct of the bursa. The duct of the seminal receptacle (Dsr), very small and wider than long, joins the oviduct just before

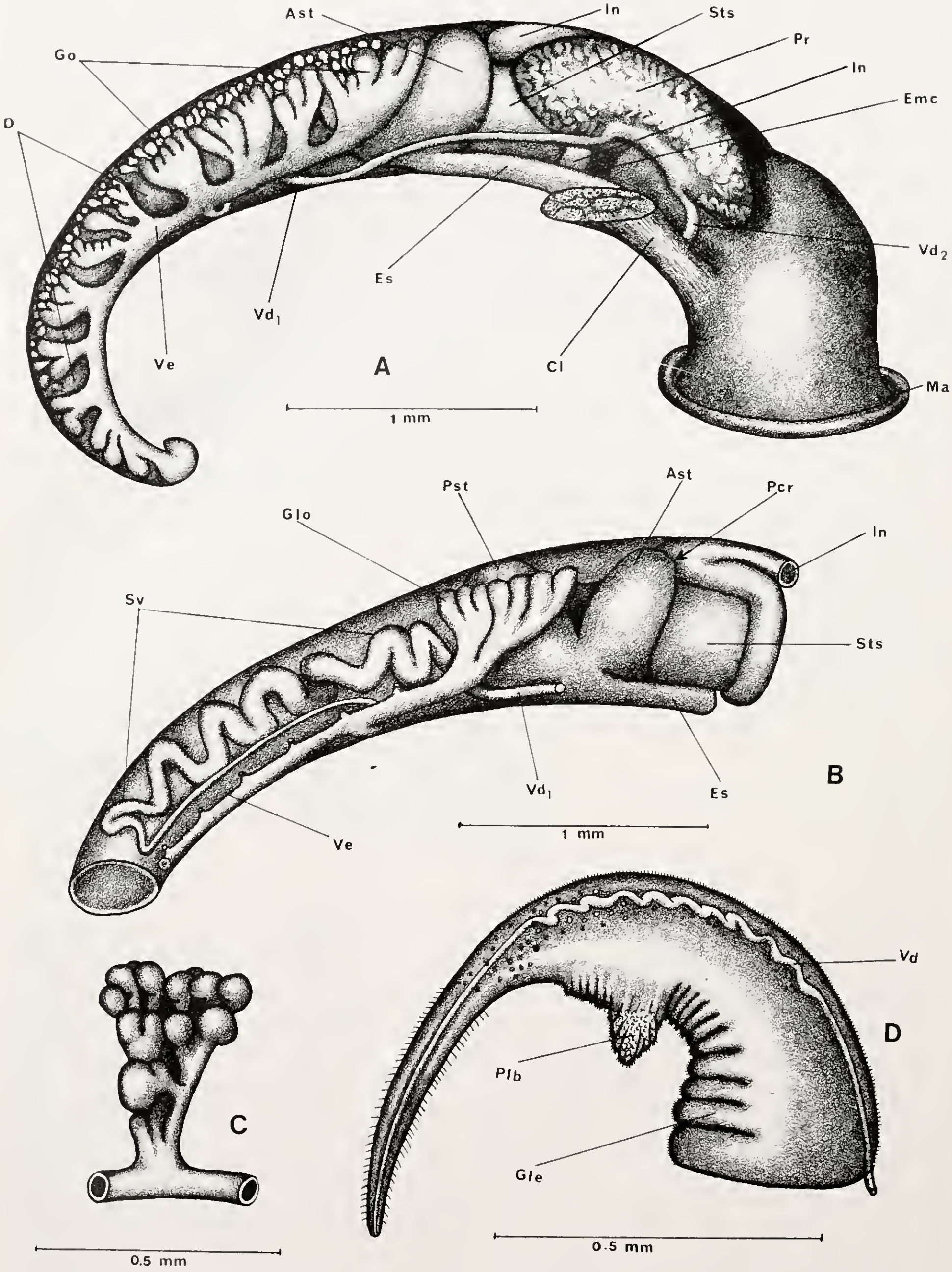
Ast
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FIGURE 4. Male reproductive system. A: Male oriented as the female in Figure 2A. The kidney tissue was removed to reveal the style sac (Sts). B: Gonad with most gonadal lobes removed to reveal the position of the seminal vesicle (Sv). C: An enlarged view of one gonadal lobe with numerous sperm-producing pouches. D: The penis. Abbreviations are: Ast, anterior chamber of the stomach; Cl, columellar muscle; D, digestive gland; Emc, posterior end of the mantle cavity; Es, esophagus; Gle, glandular edge of the penis; Glo, gonadal lobe; Go, gonad: In, intestine; Ma, mantle collar; Pcr, pellet compressor; Plb, penial lobe; Pr, prostate; Pst, posterior chamber of the stomach; Sts, style sac; Sv, seminal vesicle; Vd, vas deferens; Vd_1, posterior vas deferens; Vd_2, anterior vas deferens; Ve, vas efferens.

the latter joins the duct of the bursa at a common opening (Ca) to the posterior end of the ciliated groove of the ventral channel (Vc). The ventral channel forms the ventral edge of the pallial oviduct with which it is in open communication. This V-shaped gutter has red-pink granules along its entire length. The morphological inter-relationships of the pallial oviduct, ventral channel, bursa and seminal receptacle characterize, in great part, the genus *Hydrobia.*

The bursa copulatrix-posterior pallial oviduct complex lies ventral to the style sac (Sts) of the stomach and the loop of the intestine circling the anterior end of the style sac. The common opening (Ca) to the ventral channel of the pallial oviduct is just posterior to the posterior end of the mantle cavity (Emc). The ratio of the length of the bursa copulatrix to the pallial oviduct is 0.15; the ratio of the length of the seminal receptacle to the length of the bursa is 0.51. The ratio of the length of the pallial oviduct to the length of the body is 0.40. The oviduct gives off a pronounced gonopericardial duct (Gpc) before reaching the pallial oviduct and diving down beneath the bursa complex. When the bursa is lifted, and rotated through 180° (Fig. 3B–D) the coiled nature of the oviduct dorsal to the bursa is readily discerned. The complex nature of this coiling is very constant within the species. A large segment of the coil is darkly pigmented (Fig. 3B–D), a condition seen in several European Hydrobiidae such as *H. acuta* and *H. ventrosa* (Radoman, 1977). *H. truncata* stores sperm within these coils, not within the seminal receptacle (as seen histologically). In some European hydrobioids, such as various species of *Pyrgula,* the seminal receptacle is a simple outpocketing from a section of the pigmented oviduct coil (Radoman, 1955, 1956). This outpocketing may represent a stage of seminal receptacle reduction leading to the condition of sperm storage in the coils as seen in *H. truncata.* The function of the seminal receptacle in *H. truncata* is not known.

Male reproductive system. Dimensions of organs are given in Table II. As in the female, the gonad is relatively long, 2.99 mm (56% of total body length). It extends anteriorly over the stomach, and posteriorly to the tip of the digestive gland. There are usually 6 to 8 distinct anterior gonad branches and a variable number of single-lobed posterior branches. The 3-dimensional branching pattern of the gonad (Fig. 4C) is similar to that of *Pomatiopsis* (Davis, 1967, plate 12). As in *Pomatiopsis* (Davis, 1967) and other pomatiopsids (Davis, 1979), the seminal vesicle branches off the vas efferens between $\frac{1}{2}$ and $\frac{1}{3}$ the way along the gonad (usually under the fourth branch), and coils behind the stomach (Fig. 4A, B). However, in *H. truncata* the vas deferens extends straight back 1 mm along the gonad and then doubles back upon itself to swell and coil (Fig. 4B). This is quite unlike the condition in *Pomatiopsis* where the coiling begins just anterior to where the vas deferens branches off the vas efferens (Davis, 1967, plate 12).

The prostate measures 1.2 mm long (22% of body length) and has the same glandular nature as in *Pomatiopsis* (Davis, 1967). The tubular collecting duct of the prostate is devoid of glands. The vas deferens enters the prostate about 0.25 mm from its posterior end, coincident with the end of the mantle cavity (Fig. 4A). The anterior vas deferens leaves the prostate 0.37 mm from its anterior tip. The positions of entrance and exit of the vas deferens from the prostate are somewhat intermediate between those of *Pomatiopsis* (Davis, 1967) and *H. totteni* (Davis, 1966). After extending down along the mantle wall to enter the head region, the vas deferens coils (more so than in Davis, 1966, Fig. 3) upwards to enter the penis.

The penis has one duct and a glandular lobe on the concave curvature (Fig. 4D). Its form is similar to that of *H. totteni* (Davis, 1966, Fig. 5), but it differs in

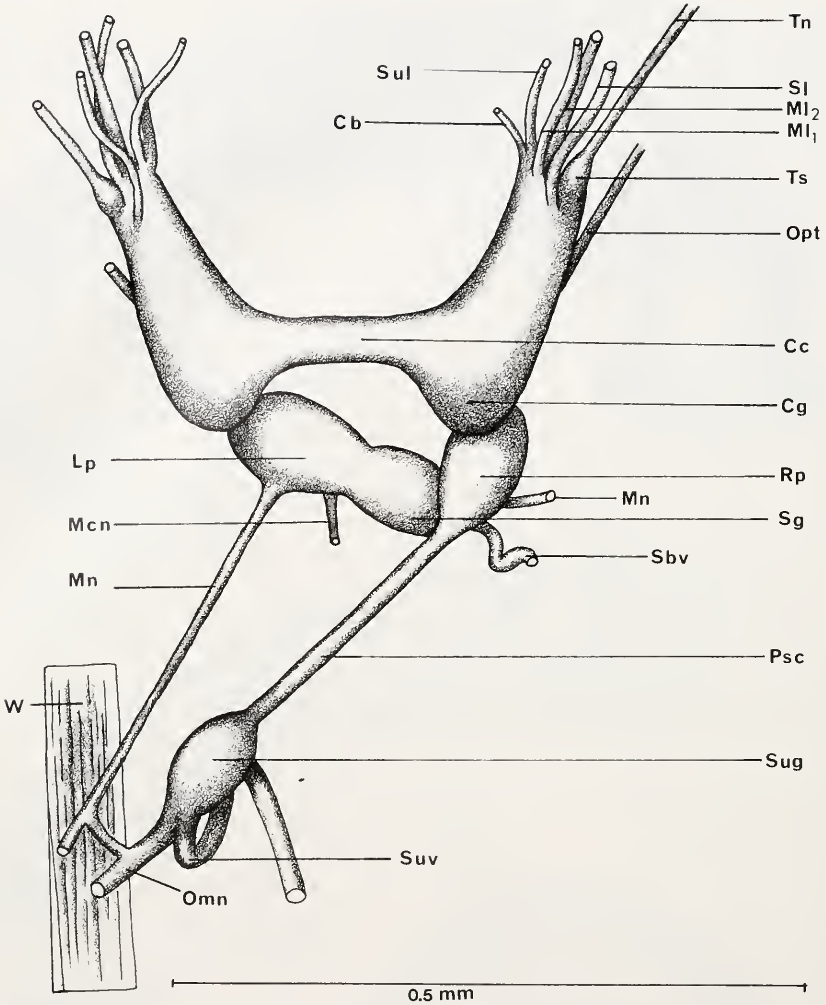

FIGURE 5. Dorsal nervous system. Abbreviations are: Cb, cerebro-buccal connective; Cc, cerebral commisure; Cg, cerebral ganglion; Lp, left pleural ganglion; Mcn, mid columellar nerve; Ml_1, median labial nerve 1; Ml_2, median labial nerve 2; Mn_1, mantle nerve 1; Mn_3, mantle nerve 3; Omn, osphradiomantle nerve; Opt, optic nerve; Psc, pleuro-supraoesophageal connective; Rp, right pleural ganglion; Sbv, subvisceral connective; Sg, suboesophageal ganglion; Sl, supralabial nerve; Sug, supraoesophageal ganglion; Sul, sublabial nerve; Suv, supravisceral connective; Tn, tentacular nerve; Ts, basal swelling of tentacular nerve; W, left cephalic wall.

several important respects. Penial folds line the entire concave surface of the verge from the lobe to the base of the verge, whereas in *H. totteni* the folds end far from the end of the base. The penial lobe is not a smooth, simple appendage as in *H. totteni* (Davis, 1966, Fig. 5); instead, it is more triangular and has glandular folds perpendicular to the verge. The glandular lobe seems to be further out along the penis from the base in *H. truncata* compared to its position in *H. totteni*. Comparing the ratio of the length of the verge from its base to the distal edge of the lobe to the length of the verge from its base to the end of the glandular folds beyond the lobe, the ratio is 0.83 for *H. truncata* and 0.67 for *H. totteni.* The penial filament appears to be relatively longer and more attenuated than in *H. totteni.* The ciliation patterns of the verges of these two species are similar except that the entire concave surface of the verge of *H. truncata* is ciliated while that of *H. totteni* is unciliated beyond the filament. As in *H. totteni,* the vas deferens travels close to the convex surface of the verge and is frequently convoluted. There is no terminal papilla. The glands appear to be distributed over a wider area than those of *H. totteni* (contrast Fig. 4D with Fig. 5 of Davis, 1966). Two gland types, Gl$_1$ and Gl$_2$ (see Davis, 1969b) are found on the verge. G$_1$ glands, which appear as closely packed black dots, are found all over the verge, but particularly near the base of the penial filaments. Gl$_2$ glands, closely packed spheres, are usually clustered around the bends and folds of the vas deferens. As in *H. totteni,* the verge coils counter-clockwise in life.

Nervous system. The nervous system varies little from that described for other hydrobioids (Krull, 1935; Davis, 1967, 1968, 1969b; Davis and Carney, 1973, Davis *et al.,* 1976), except for degrees of concentration. Lengths of neural structures are given in Table III. The cerebral ganglia give off the same seven nerves as in all rissoaceans (Figs. 5, 6). The base of the tentacular nerve is somewhat swollen, but not as much as in "*Lithoglyphopsis*" *aperta* (Davis *et al.,* 1976). The cerebral commisure is quite elongated. The pleuro-supraoesophageal connective is relatively long as in other species of *Hydrobia* (see Krull, 1935, Davis *et al.*, 1976). The RPG ratio (see Davis *et al.,* 1976, for formula), an index of neural concentration, is 0.547, a value quite close to those of *H. ventrosa* and *H. ulvae* (Davis *et al.,* 1976). A dialyneury connecting the osphradio-mantle nerve and mantle nerve 1 is found (Fig. 5). This has been figured in other publications on hydrobioids (Davies, 1968b; Temcharoen, 1974, unpublished Ms. thesis, Thomas Jefferson University, Philadelphia, PA). As in *Pomatiopsis* (Davis, 1967), the mid-columellar nerve is variable in origin but usually it comes off the left pleural ganglion rather than the suboesophageal ganglion.

The cerebro-pedal complex (Fig. 6), is of the usual hydrobioid type. A minor lateral nerve (P$_8$) is frequently found on the pedal ganglion. The relative lengths of the propodial ganglia, the metapodial ganglia, and their connectives to the pedal ganglia are much more similar to those of *Pomatiopsis* (Davis, 1967) than in "*Lithoghlyphopsis*" *aperta* (Davis *et. al.,* 1976). As in other hydrobioids, the

TABLE III

Measurements (mm) of lengths of neural structures of Hydrobia truncata.

Structure	No.	$\bar{X}$	s.d.	Range
Cerebral ganglion	9	0.206	0.020	0.18 –0.24
Cerebral commisure	12	0.090	0.018	0.056–0.13
Right pleural ganglion	9	0.100	0.010	0.08 –0.12
Left pleural ganglion	8	0.104	0.007	0.10 –0.12
Pleuro-supraoesophageal connective	9	0.233	0.023	0.21 –0.26
Supraoesophageal ganglion	8	0.093	0.009	0.084–0.10
Osphradial-mantle nerve	6	0.058	0.016	0.04 –0.08
Suboesophageal ganglion	9	0.097	0.013	0.08 –0.12
Pedal ganglion	10	0.176	0.024	0.14 –0.22
Pedal commisure	13	0.040	0.013	0.036–0.06
Buccal ganglion	5	0.102	0.005	0.10 –0.11

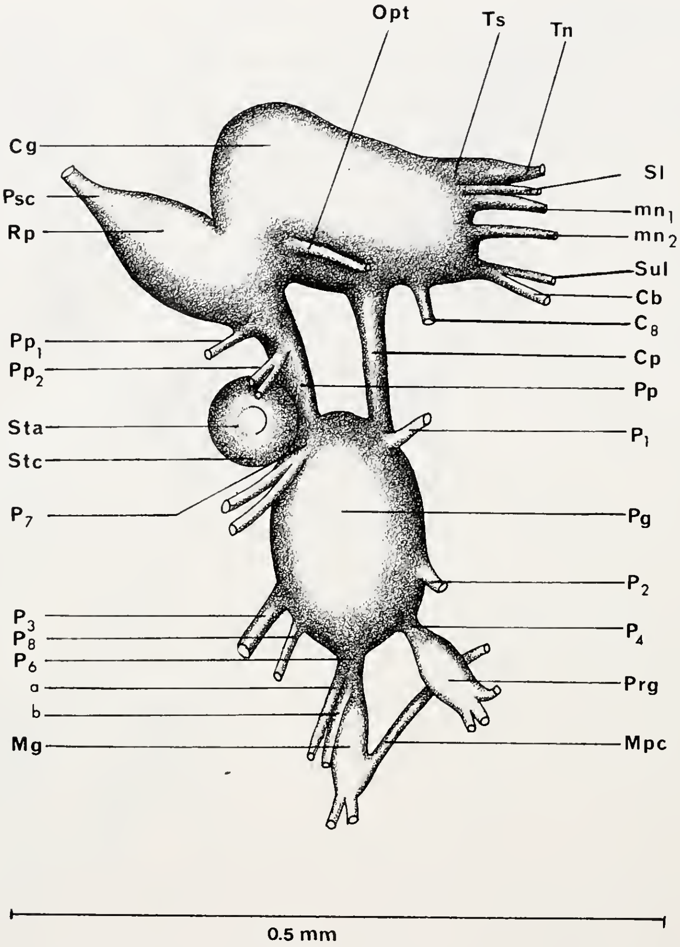

FIGURE 6. Lateral view (right) of Cerebropedal complex. Abbreviations are: a, nerve from metapodial connective; b, nerve from metapodial connective; Cb, cerebro-buccal connective; Cg, cerebral ganglion; Cp, cerebropedal connective; C_8, cerebro-tensor nerve; Mg, metapodial ganglion; Mn_1, media labial nerve 1; Mn_2, median labial nerve 2; Mpc, metapodial commissure; Opt, optic nerve; Pc, pedal commissure; Pg, pedal ganglion; P_p, pleuro-pedal connective; Prg, propodial ganglion; P_1, lateral retractor nerve; P_2, nerve to antero-ventral wall of the pedal haemocoel; P_3, major lateral nerve; P_4, propodial connective; P_6, metapodial connective; P_7, dorso-lateral pedal nerve; P_8, minor lateral nerve of the pedal ganglion; Pp_1, lateral nerve 1; Pp_2, lateral nerve 2; Psc, pleuro-supraoesophageal connective; Rp, right pleural ganglion; Sl, supralabial nerve; Sta, statolith; stc, statocyst; Sul, sublabial nerve; Tn, tentacular nerve; Ts, basal swelling of the tentacular nerve.

origin of the two nerves of the metapodial connective is variable. The connection between the two metapodial ganglia is seen as figured in *Oncomelania minima* (Bartsch) (Davis, 1969b). All the ganglia except the suboesophageal and supraoespophageal are pigmented.

Parasites. Loosely attached epibionts, similar in appearance to ciliated protozoans figured in Davis and Carney (1973), were found on the outside head-

TABLE IV

Analyses of nine shells from living specimens of Hydrobia truncata *from Stone Harbor, N. J. (measurements in mm).*

Shell trait	Females		Males	
	$\bar{X}$	s.d.	$\bar{X}$	s.d.
Length	3.11	0.105	2.81	0.087
Width	1.70	0.071	1.52	0.036
Length of body whorl	2.02	0.042	1.84	0.068
Aperture length	1.24	0.034	1.14	0.044
Aperture width	0.873	0.044	0.804	0.029

foot region of many individuals. Our dissection records show that an unidentified trematode parasitized 16% (33/206) of the Stone Harbor snails studied.

Shells. It may not be possible in every instance to distinguish among northeastern U. S. A. species of *Hydrobia* by examining shells under the dissecting microscope. They are all small (< 4.5 mm), ovate to ovate-conic, smooth, and brown to yellow brown. The umbilical area varies from closed to rimate, moderately open, or pronouncedly open. There is great intra- and interpopulation variability in size and shape. The following descriptions are a partial help for species identification.

Hydrobia truncata. Specimens of *Hydrobia truncata* from Stone Harbor (Fig. 7E–I) are sexually dimorphic (Table IV). Females were significantly larger than males. This could, in part, account for the separated clusters of individuals along the y axis in Figure 8.

The shell is more turreted than ovate-conic (contrast Figs. 7C and 7A) due to a low rate of whorl expansion (defined in Raup, 1966). By comparing the width of the fourth whorl (adapical from aperture)/length of the last three whorls, it is evident that the fourth whorl of *H. truncata* is proportionally larger than that of *H. salsa* or *H. totteni* (Figs. 7A, C; 8). The shell is decollate; the apical one to two whorls are not present on half grown to mature specimens. This decollation contrasts with the various stages of erosion of whorls seen in *H. totteni.* Decollation or erosion has not been seen in, or reported for *H. salsa.*

The whorls are very convex and the suture correspondingly deep. The length of the body whorl is less than that of *H. salsa* or *H. totteni* (Table I). The umbilicus is rimate (*i.e.* very small and slit-like). The length is variable depending on the population, ranging from 2.50 to 3.32 mm (length of last three whorls). Parasitized individuals may have a total length (rare cases with all whorls present; only at Stone Harbor) of 4 mm while unparasitized individuals are less than 3.5 mm in total length.

Hydrobia salsa. The shells are ovate-conic (Fig. 7B) and have a high rate of whorl expansion. The proportion of the fourth whorl compared to the length of the last three whorls is smaller than *H. truncata* (Fig. 8). The shell is entire with 5.0 to 5.5 whorls. The whorls are very convex and the sutures very deep. The length of the body whorl is greater than that of *H. truncata* and *H. totteni.* The umbilicus is very wide and pronounced. The shell length of the paratypes ranges from 3.0 to 3.2 mm (last three whorls); given all whorls the length may be 3.39 to 3.51 mm.

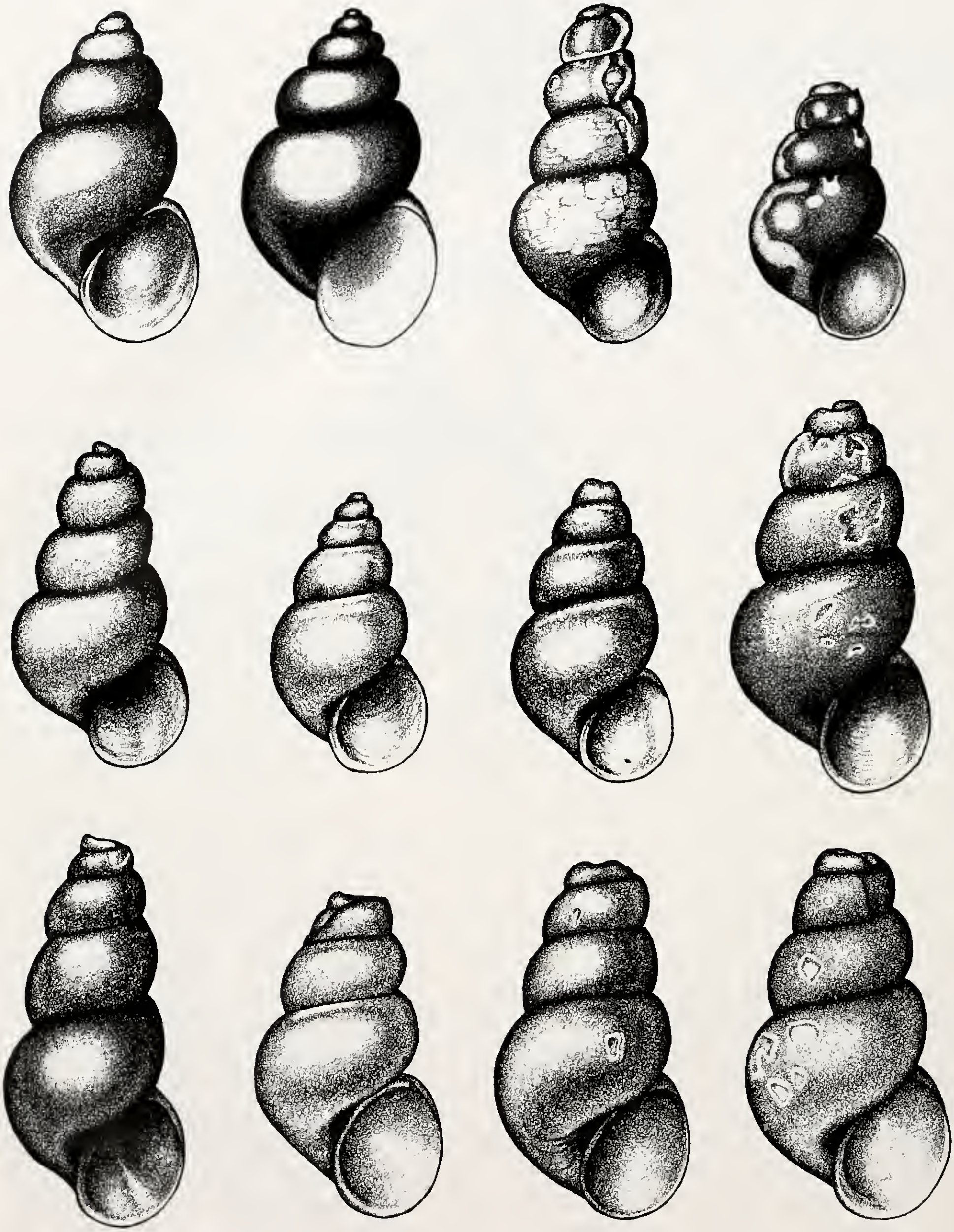

FIGURE 7. Left to right, top row to bottom, shells of *Hydrobia totteni* (A), *H. salsa* paratype (B), *H. truncata* paratypes (C, D), *H. truncata* from Stone Harbor (E–I), *H. truncata* from Salt Pond (J–L). The shell length of A is 3.2 mm; other shells are drawn to the same scale.

Hydrobia totteni. The shells vary from ovate to ovate-conic (Fig. 7A) and have a high rate of whorl expansion as discussed for *H. salsa* (Fig. 8). They vary from uneroded to severly eroded so that often only three whorls are seen; the degree of erosion varies with the population. When shells are entire, 5.0

whorls are seen. The convexity of whorls is greatly variable in a population, from slight to pronounced; sutures are correspondingly shallow to moderately deep. The length of the body whorl is greater than that seen in *H. truncata,* less than that seen in *H. salsa* (Fig. 9). The umbilical opening is rimate to moderately open. The shell length ranges from 2.52 to 3.07 mm, depending on the population; given all whorls, the length of the largest specimens ranges from 2.93 to 3.39 mm.

The shells of the seven populations are compared for five variables (Table I). Considering size alone (column 1), specimens of *H. truncata* from Salt Pond are the largest. However, as seen, absolute size varies considerably among populations of a species. Accordingly, the species cannot be simply identified on the basis of size. Specimens of *H. salsa* have the widest shell per size (column 2). Again, there is considerable variation among populations of a species. There is a clear difference between *H. truncata* (all populations), and *H. salsa* and *H. totteni* in length of body whorl per size (column 3). There is a difference between *H. truncata,* and *H. salsa* and *H. totteni* in length of aperture per size (last column). Considering the above descriptions and Table I, there is much more similarity between shells of *H. totteni* and *H. salsa* than between either of these and *H. truncata.* Further differences among populations and species are seen in Figures 8–10.

The ratio of length of body whorl is plotted against length of the last three whorls for shells of six populations (Figs. 9, 10). The regression for pooled data from the six populations was highly significant ($P = 0.0001$) yielding a formula of $y = 0.34$ to $0.58x$. Considering each population separately, the regression was

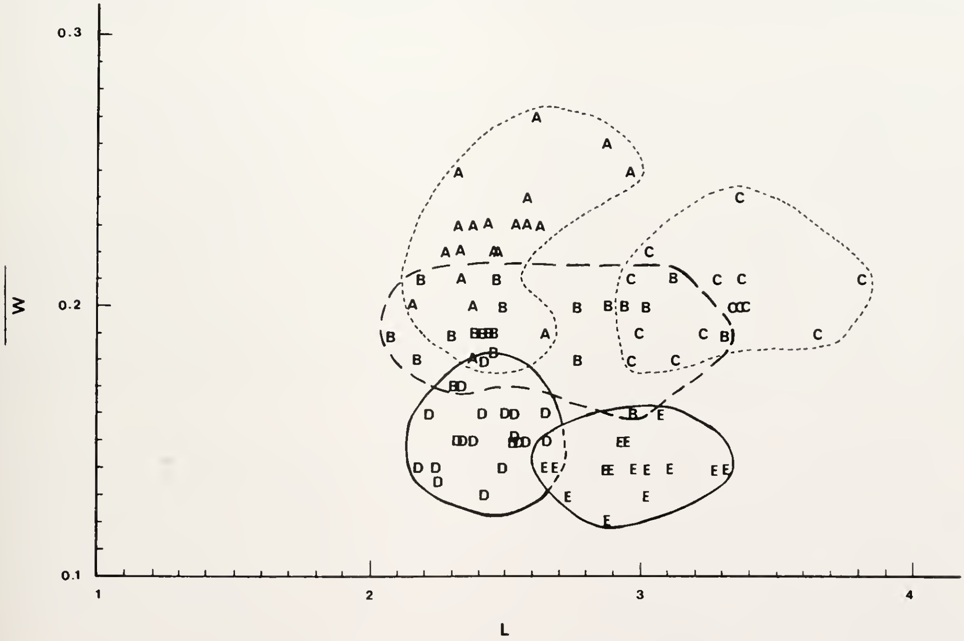

FIGURE 8. Analysis of shells from five populations involving the relationship of the width of the 4th whorl (adapical from the body whorl) divided by the length of the last three whorls (W) to the length of the last three whorls (L) in mm. A: *H. truncata,* paratypes: B: *H. truncata,* Stone Harbor; C: *H. truncata,* Salt Pond; D: *H. totteni,* Martha's Vineyard; D: *H. salsa* paratypes.

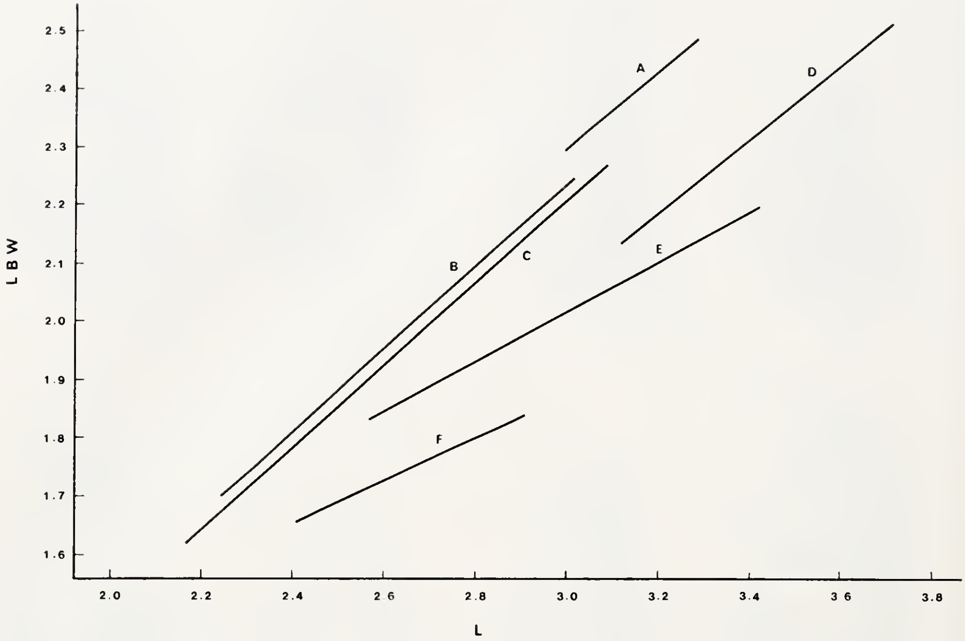

FIGURE 9. Computer plotted regressions of length of body whorl (LBW) on length of the last three whorls (L) (mm). A: *H. salsa*, paratypes; B: *H. totteni*, Martha's Vineyard; C: *H. totteni*, Provincetown; D: *H. truncata*, Salt Pond; E: *H. truncata*, Stone Harbor; F: *H. truncata*, paratypes; Towne Pt.

highly significant ($P = 0.0001$) for both populations of *H. totteni*, and *H. truncata* from Towne Point and Stone Harbor; significant ($P = 0.001$) for *H. truncata* from the Salt Pond; not significant for *H. salsa* ($P = 0.80$) (due to the scatter for only five data points). Each regression is shown in Figure 9.

Paratype specimens of *H. truncata* (Towne Point) and Stone Harbor specimens of *H. truncata* have a significantly greater slope than do the other populations (but not significantly different between themselves). Of the 15 possible cross comparisons for y intercept there were 5 significant differences ($P < 0.001$). These differences involved the two populations of *Hydrobia totteni* (Martha's Vineyard and Provincetown) compared with *H. truncata* from Stone Harbor and from Towne Point; there was a significant difference between the Salt Pond and Towne Point populations of *H. truncata*.

The scatter of data points for *H. salsa* coupled with observations on the rate of whorl increase and relative size of the fourth whorl (Fig. 8) indicated that *H. salsa* was not allometrically different from *H. totteni* but was larger than *H. totteni*. Accordingly, the data for *H. salsa* and the two populations of *H. totteni* were pooled and compared with the pool of data for these three populations considered to be *H. truncata*. The two regressions are significantly different (Fig. 10). The y intercepts and slopes are very significantly different ($P = 0.0001$).

H. salsa and *H. totteni* have a more rapid whorl expansion rate than do populations of *H. truncata* (Figs. 7, 8). The proportionally narrow fourth whorl more clearly separates *H. salsa* and *H. totteni* from *H. truncata* while again, *H. salsa* is seen to be larger than *H. totteni* in overall size (Fig. 8). Distribution of data along the x axis indicates that the Stone Harbor and Cape Cod populations of *H. truncata* are quite similar except in size. The pattern of distribution of data along the y axis shows that some individuals of the Cape Cod population, and many individuals of the type population of *H. truncata* vary considerably in the width of the fourth whorl. Shells with the wider fourth whorls are rather cylindrical. As decollation beyond the fourth whorl of mature specimens is the rule, the trend in the data may indicate that in some specimens the larger fourth whorl reflects an advanced whorl stage in growth. This does not, however, account for the spread in data at a size of 2.35 mm.

DISCUSSION

This study presents the morphological groundplan of *Hydrobia truncata* as the starting point for future studies of *Hydrobia* world-wide. Three factors presented here have not been considered by previous students of *Hydrobia*, namely: the positional relationships of organs within the body relative to each other and to important topographical features such as the posterior end of the mantle cavity, the anterior end of the style sac, etc; the comparative size of different organs, for example, length of the bursa copulatrix to the length of the pallial oviduct; size and scaling such as discussed by Pilbeam and Gould (1974). These factors have been used with positive results in studies of the Pomatiopsidae (Davis and

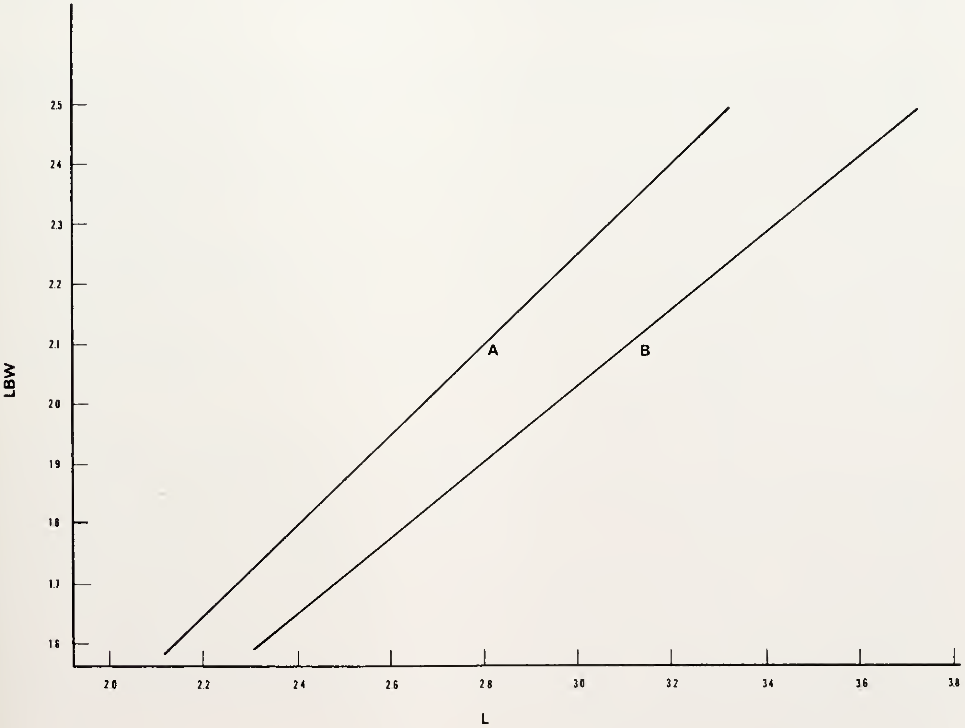

FIGURE 10. Computer plotted regressions of length of body whorl (LBW) on length of the last three whorls (L) (mm). A: *H. salsa* and the two populations of *H. totteni*; B: the three populations of *H. truncata*.

Table V

Eleven morphological differences between Hydrobia truncata *and* H. totteni.

H. truncata	*H. totteni*
1. Gill filament number 19–25	9–18 (depending on maturity)
2. Pallial appendage projecting and prominent	Button-like and hidden inside mantle edge
3. Penial appendage triangular and with folds	Appendage club-like, without folds
4. Penial appendage near mid-penis: length of gland area in front of appendage to length of penis base to distal edge of appendage: 0.20–0.30	Penial appendage near base of penis: ratio: 0.45–0.60
5. Glands widespread throughout penis	Glands concentrated near base of filament
6. Prostate measures 1.2 × 0.42 mm	0.67 × 0.24 mm
7. Vas deferens leaves prostate 0.37 mm posterior to anterior end of prostate	Leaves anterior end of prostate
8. Vas deferens greatly coiled before entering penis	Not coiled
9. Length of body whorl/length of last three whorls: 0.74–0.75	: 0.67–0.69
10. Shells decollate	Many eroded but not decollate
11. Width of 4th whorl/length of last three whorls: 0.17–0.27	: 0.12–0.17

Carney, 1973; Davis *et al.*, 1976; Davis, 1979). This study has also shown that *Hydrobia truncata* is found in localities where previously only *H. totteni* was thought to exist, namely from New Jersey to at least Massachusetts.

While the three populations of *Hydrobia totteni* differ little in shell length or in the regression analyses, there was considerable variability among populations of *H. truncata* both in shell length (Table I) and in the regressions of L1/L3 (Fig. 9). As one moves from Maryland to Massachusetts the size increases and the slope of the regression increases so that specimens of *H. truncata* from Massachusetts differ significantly from those from Maryland. The allometry of Massachusetts populations of *H. truncata* equals that of *H. totteni* only when specimens of *H. truncata* reach a size at maturity that is considerably larger than that of *H. totteni* (22% larger).

H. salsa is a larger species than *H. totteni* (about 19% larger) with a similar rate of whorl increase (Fig. 9) and probably the same allometry (Figs. 8–10). There are clearly two different classes of allometry considering the plots of L1/L3, one including *H. totteni* and *H. salsa,* the other *H. truncata.* Of particular interest is the change in shell size, and slope of L1/L3 in populations of *H. truncata* from south to north. We do not know why this occurs and the question remains: will other populations of *H. truncata* fit this pattern? It is likewise of interest that where the three species can be found in the same state (Massachusetts) the shell allometry regarding L1/L3 is the same for the three species. In Massachusetts, however, the species are separable on the basis of size, degree of umbilication, and rate of whorl increase.

Hydrobia truncata and *H. totteni* apparently differ by 11 character states (Table V), three of which are shell characters. However, we are far from satisfied that all of these character state differences will be maintained when numerous populations from a given environment are studied. For example, while the position and shape of the penial lobe has been constant in the populations we studied, we are not convinced that interpopulation differences do not exist, and that conditions intermediate to those presented here will not be found. The specimens of *Hydrobia totteni* we examined from Wild Harbor River were sent to us unrelaxed and preserved. The penis type corresponded to that illustrated by Davis (1966). However, the penial lobes of some specimens were not pendulent, but rounded lappets. The shape was not triangular and the lobe did not have the folds described for *H. truncata.* The pallial appendage was not a large projecting tubercle as in *H. truncata,* but an insignificant button of tissue hidden within the edge of the mantle. This feature was previously overlooked (Davis, 1966). Further anatomical comparisons were not made because we have based our work on living, unpreserved specimens, and because we had too few specimens to examine all variables. A thorough morphological study of living specimens of *H. totteni* is needed before more can be said about relationships between these species.

We see in the shells and penial anatomy of *H. truncata* and *H. totteni* a problem similar to one we see in Muus (1963) for *H. ventrosa.* He figured two

TABLE VI

Comparison among five species of Hydrobia *using nine morphological characters. * Krull, 1935.*

Character and state	Taxa				
	H. truncata	*H. totteni*	*H. neglecta*	*H. ulvae*	*H. ventrosa*
1. Pallial appendage					
Filament	–	–	?	+	?
Projecting tubercle	+	–	?	–	?
Hidden node	–	+	?	–	?
2. Penis tip					
Long filament	+	+	–	–	+
Pointed, no filament	–	–	–	+	–
Blunt, no filament	–	–	+	–	–
3. Penis with penial lobe	+	+	–	–	+
4. Snout with anterior pigment bar	–	–	+	+	–
5. Tentacles with pronounced terminal pigment streak	+	+	+	+	–
6. Length of bursa/length of pallial oviduct	0.15	?	?	0.07*	0.20*
7. Length of seminal receptacle/ length of bursa	0.51	?	?	0.75*	0.55*
8. Position of bursa					
Projecting posterior to albumen gland	–	?	?	+*	+*
Not projecting posterior to albumen gland	+	?	?	–	–
9. Radula					
No. rows teeth	37–45	31–43	38–45	42–52	38–44
Length (mm)	0.5–0.7	0.5–0.6	0.6–0.8	0.8–1.2	0.7–0.8

individuals: "A" with a conical shell similar to *H. truncata* had a shell of 7 whorls, the length of the body whorl was 2.2 mm, the length of the last three whorls was 3.6 mm, and the width of the fourth whorl (counting from the base) divided by the length of the last three whorls was 0.23; "B" was ovate conic like *H. totteni* and had corresponding data of 1.44, 2.64, and 0.16 mm. The "A" form groups with our salt pond population of *H. truncata* and the "B" form groups with the *H. totteni* population. The position of the penial lobe for "A" is similar to that of *H. truncata;* the position in "B" is similar to that in *H. totteni.* This poses several questions. Are the two types of individuals referred by Muus (1963) to *H. ventrosa* the same or different species? Is there variation in the position of the penial lobe in *H. ventrosa* and *H. totteni?* What accounts for the differences in shell types A and B if both really belong to the same species? The penial lobe illustrated by Muus resembles that of *H. totteni* illustrated by Davis (1966). Is that interpretation correct? Is the large form of *H. ventrosa* due to parasitism, as we see in larger specimens of *H. truncata?* If such extremes in shell shape and penial structure can be proved to exist in *H. ventrosa* does this indicate that *H. truncata* and *H. totteni* are the same species? Do *H. ventrosa* and *H. neglecta* have a pallial appendage?

Considering the limited data we do have, the three species of *Hydrobia* from eastern North America apparently are separate ecologically and morphologically. Confirmation rests with further studies of numerous populations.

Considering *H. salsa,* there are no soft-part morphological data. Shells are larger than those of *H. totteni,* much more ovate-conic than *H. truncata.* According to Mazurkiewicz (1972) *H. salsa* inhabits environments with 0.4 to 20‰ salinity; the lower level of salinity tolerance for adults is 0.2‰ and for veligers 1.0‰. Young emerged from capsules of 115–125 μm; the egg diameter is 95 to 100 μm. *H. salsa* has free floating veligers. The question is, do populations studied by Mazurkiewicz correspond to the types?

Considering *H. totteni,* there are limited morphological data (Davis, 1966). Shells are smaller than those of *H. salsa* but of the same general shape. The shells are much less umbilicate than those of *H. salsa.* The species apparently lives in a wide range of salinities. Those reported from New England, U. S. A., lived in 32‰ or more (Mazurkiewicz, 1972). They are most dense on mid-intertidal sand flats of a Nova Scotia, Canada, estuarine marsh with salinities of 27.6 to 29‰ (Wells, 1978). Young emerged from egg capsules of 290 to 310 μm diameter. The diameter of the eggs was 150 to 160 μm. The lower limit of salinity tolerance was 5‰ for adults and 2‰ for emerging young (Mazurkiewicz, 1972).

We have considerable morphological data for *H. truncata.* The species apparently lives in high salinity (32‰) in high intertidal marsh pools. Snails from Stone Harbor brought into the laboratory and maintained in Petri dishes with substrate from their habitat laid eggs on shells and the bottom of the dish. The single egg capsules on the shells were 294 to 308 μm in diameter. A number of double egg capsules bound in a single membrane were observed on the bottom of the dishes. Crawling young emerged from the egg capsules. Salinity tolerances are not known.

We have much more morphological data for *H. ventrosa* than for *H. totteni* (Krull, 1935; Muus, 1963; Radoman, 1977; Bishop, 1976) yet we still do not know enough to enable an adequate comparison with *H. truncata.* The adults can easily tolerate salinities up to 35‰ but perfer quiet waters in estuaries where the salinities range from 5 to 24‰ (Muus, 1967). Bishop (1976) records salinity range 6 to 29‰. The egg capsule is 260 μm in diameter (Thorson, 1946) and thus smaller than that of *H. totteni.* The young hatch in the crawling state.

A limited comparison can be made between populations of *H. truncata* and *H. totteni* of eastern North America, and *H. neglecta* Muus, *H. ulvae* (Pennant) and *H. ventrosa* (Montagu) of northern Europe (Table VI). The North American species are clearly different from *H. ulvae* and *H. neglecta* in penis morphology and snout pigmentation. *H. ulvae* has a significantly larger radula than do other species. If data given by Krull (1935) are reproducible, and variation in length of the bursa copulatrix is small as in *H. truncata* (Table II), then *H. ulvae* has a relatively much smaller bursa than do *H. truncata* and *H. ventrosa;* the seminal receptacle of *H. ulvae* is relatively much larger in comparison to the bursa (Table VI).

Given the limited data available, *H. truncata* and *H. totteni* more closely resemble *H. ventrosa* than other species. Resemblance includes penis morphology, snout pigmentation, and radular length (Table VI). The similarity of *H. ventrosa* to *H. totteni* has been discussed previously (Muus, 1963; Davis, 1966). Bishop (1976) reexamined *H. ventrosa* and found that *H. ventrosa* and *H. totteni* did not differ in hypertrophy of cilia on the left tentacle, structure of radula (scanning electron microscopic studies showed Muus' drawings to be in error), or pigment streak on the tentacle (the streak is present in *H. ventrosa* to varying degress in different populations).

A number of fundamental questions concerning morphology are unanswered for northern European *Hydrobia.* Do these species (*H. neglecta, H. ventrosa*) have a pallial filament or tubercle? How many gill filaments do they have? What is the exact morphology and position of the penial appendage? Are the oviduct coils pigmented? Is sperm stored in the seminal receptacle or in the coils of the oviduct? What is the length of the albumen gland relative to the length of the entire pallial oviduct? Does the gonad fill the digestive gland and overlap the stomach?

Considering the few morphological details of the female reproductive system of *H. ventrosa* given by Krull (1935) and Radoman (1977; material collected from Holland) it is not entirely clear what the situation is. There is apparent agreement that the length of the bursa copulatrix per length of the pallial oviduct is 0.20 and thus relatively larger compared with that of *H. truncata* (0.15). The length of the albumen gland compared to total pallial oviduct length is less in *H. truncata* (0.38–0.40 contrasted with 0.49 given by Radoman; not shown by Krull). The position of the bursa of *H. truncata* is anterior to the posterior end of the pallial oviduct; it is the same for *H. ventrosa* as portrayed by Radoman; it is posterior to the end of the pallial oviduct as shown by Krull. The shape of the penial lobe of *H. ventrosa* shown by Krull and Radoman is triangular, *i.e.* similar to that of *H. truncata.*

It is thus evident that a thorough quantitative morphological reevaluation of *H. ventrosa* is needed. It is hoped that future workers will provide measurements for major organs and for the length of the body, provide statistics for these measurements, illustrate organ systems relative to other body organs, provide statistics on shell parameters that include the length of body whorl, length of the last three whorls, L1/L3, and width of the fourth whorle per length of the last three whorls—in addition to others that might be made. The exact position of the penial appendage relative to the glandular fields of the penis is needed along with morphological details of the appendage.

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SUMMARY

1. Detailed morphological data for *H. truncata* are given as a standard for comparing species of *Hydrobia* worldwide, stressing the importance of quantitative data, scaling and allometry. The need for careful examination of all organ systems is stressed. Useful data for comparing species have been derived, in order of importance, from the female reproductive system, male reproductive system including details of penial filament, appendages, glands; radula, mantle-pallial structures, head pigment patterns, shell allometry.

2. Three populations of *Hydrobia truncata* from Maryland, New Jersey, and Massachusetts respectively were compared morphologically and ecologically with *H. totteni* and *H. salsa* of Massachusetts and *Hydrobia* from northern Europe. Three species of *Hydrobia* inhabit the same geographic region in Massachusetts.

3. *H. truncata* differs from *H. totteni* morphologically and ecologically. Shell size and allometry separate the species, as well as differences in eight anatomical character states. However, the complete anatomy of *H. totteni* has yet to be done.

4. *H. truncata* apparently increases in size and increases the length of body whorl relative to overall length from south to north. Allometry of Massachusetts *H. truncata* equals that of *H. totteni* when *H. truncata* exceeds *H. totteni* in size.

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PHYSICAL AND TEMPORAL FACTORS INFLUENCING THE FREEZING TOLERANCE OF THE MARINE SNAIL *LITTORINA LITTOREA* (L.)[1]

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Temperature is an important physical factor influencing freezing tolerance. Changes in temperature either before, during, or after a freezing stress can alter freezing tolerance. These temperature effects, moreover, may be dependent on time (Asahina, 1966; Levitt, 1972; Christophersen, 1973; Nowosielski-Slepowron and Strevens, 1973; McGann, Kruuv, Frim and Frey, 1975; Palta, Levitt, and Stadelmann, 1977). The increase in the freezing tolerance of marine mollusks following an exposure to low temperatures (0–5° C) prior to a freezing stress is dependent on time, with an exposure period of 2 to 3 weeks necessary to fully increase the freezing tolerance (Theede, 1972; Murphy and Pierce, 1975). However, the influence of temperature and time on the extent of freezing damage that occurs during and after a freezing stress has not been examined in marine mollusks.

Differences in freezing and thawing rates can also influence the freezing tolerances of a variety of organisms and cells (Mazur, 1970; Finkle, Pereira, and Brown, 1974; Malek and Bewley, 1978). When the freezing rate exceeds a critical value characteristic of a certain cell type, increases in freezing injury will occur. This increase in freezing injury is due to the formation of intracellular ice (Mazur, 1977). The influence of thawing rates on freezing tolerance is dependent on freezing rates. Slow thawing causes less freezing damage than rapid thawing when the freezing rate is below the critical value. In contrast, at freezing rates above the critical value, slow thawing causes greater injury than rapid thawing (Rapatz, Luyet, and MacKenzie, 1975; Mazur, 1977; Malek and Bewley, 1978). The freezing temperature, the temperature of the thawing medium, and body weight are three major physical factors which can influence the freezing and thawing rates of marine mollusks. However, neither the effects of these physical factors on the freezing and thawing rates of marine mollusks, nor the influence of freezing and thawing rates on their freezing tolerance have been examined.

Since little is known about the influence of physical and temporal factors on the freezing tolerance of marine mollusks, the purpose of this research was to define the effects of these factors on the freezing tolerance of the marine snail *L. littorea* (Mollusca: Gastropoda: Prosobranchia). *L. littorea* was chosen for the study because it is an intertidal animal which is exposed to subfreezing temperatures during the colder winter months and, in addition, has a relatively high degree of freezing tolerance (Kanwisher, 1955; Somme, 1966; Murphy, 1979).

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MATERIALS AND METHODS

Animals

Specimens of *Littorina littorea* were collected from a rocky intertidal area at Avery Point in Groton, Connecticut, between June 1, 1978, and January 15, 1979. Prior to use in experiments, the snails were held for a period lasting 2 to 4 weeks in a 190-liter (50-gallon), temperature controlled aquarium filled with 30‰ sea water at 5° C (±1° C). All sea water was collected from Long Island Sound, and the salinity was adjusted either by concentrating with artificial sea-salts (Instant Ocean, Aquarium Systems, Inc.) or by diluting with deionized water.

Calculation of freezing and thawing rates

Freezing rates. Specimens of *L. littorea* were exposed to various subfreezing temperatures by being placed into one of nine glass jars (1-liter capacity) partially submerged in the enclosed cooling chamber of a circulating refrigeration unit. The unit pumped an antifreeze solution around the jars and, with the chamber sealed, regulated air temperatures within the jars down to −20° C (±0.2). The snails were frozen in air since this best simulates natural conditions where freezing occurs following aerial exposure at low tides. The changes in the body temperature of each snail exposed to a particular temperature were continuously monitored (±0.1° C) with a copper-constantan thermocouple probe inserted into the visceral tissue. A hole was drilled through the shell to insert the probe, and the probe was held in place with dental wax. The thermocouple probe was connected to a recording thermometer, and the output of the thermometer was displayed on a strip chart recorder.

A typical freezing curve of a 1.51-g specimen of *L. littorea* exposed to a freezing temperature of −10° C is shown in Figure 1. Point A represents the temperature and time at which tissue ice formation occurred (*i.e.* supercooling point). The increase in temperature between points A and B resulted from the heat of fusion released as the tissue water was converted to ice, while point C is the temperature and time at which thermal equilibrium was attained (*i.e.* temperature change was less than 0.05° C/min). The rate of temperature change between points B and C was used to calculate the freezing rate of the tissue.

Thawing rates. Following exposure to a given subfreezing temperature, each specimen of *L. littorea* was removed from the chamber and immediately transferred into 120 ml of 30‰ sea water at temperatures between 0 and 25° C. The snails were thawed in sea water since this type of thawing usually occurs in the field during the return of sea water at high tides. The temperature changes occurring during the warming procedure are also shown in Figure 1. Following the exposure to −10° C, the snail was placed into 120 ml of 30‰ sea water at 4° C. The rate of temperature change between points D and E was used to calculate the thawing rate. Point E is the temperature 0.1° C above the freezing point of the tissue fluids. The non-linear portion of the curve near the freezing point is due to the heat absorbed as the ice is converted back into water.

Physical factors influencing freezing and thawing rates

Freezing rates. The influences of body weight and freezing temperature on the freezing rates of *L. littorea* were examined. To determine the effect of body

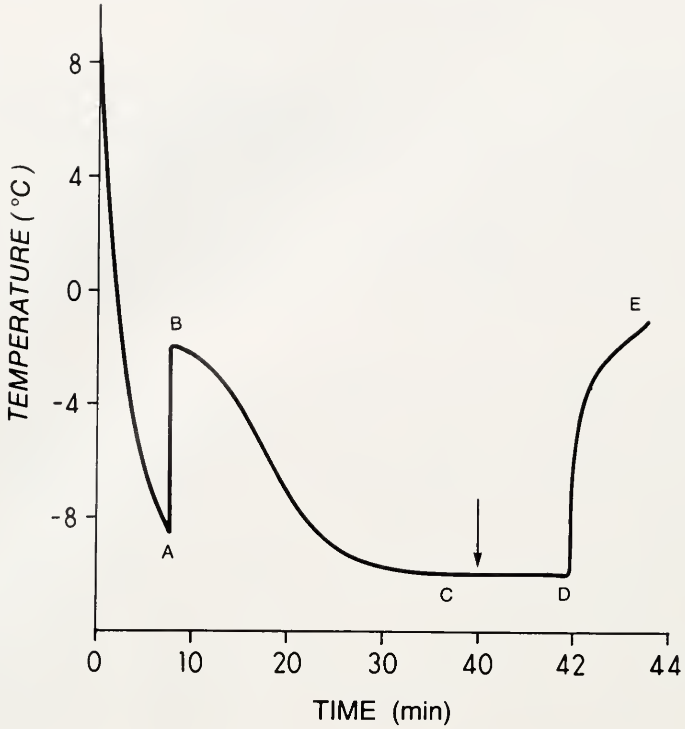

FIGURE 1. Temperature changes of 1.51-g specimens of *L. littorea* frozen in air to −10° C and thawed in 4° C sea water. Point A represents the time and temperature at which tissue ice formation occurred. The rise in temperature between points A and B is due to the heat of fusion released as the body water was converted to ice. Point C represents the time and temperature at which thermal equilibrium was attained, while points D and E are the respective times at which thawing began and was completed. The arrow indicates the time at which the chart recorder speed was changed from 0.1 to 0.5 inch/min.

weight, snails with total wet weights (*i.e.* shell and tissue) between 0.17 and 4.44 g were exposed to a temperature of −10° C. The rates of freezing were calculated as previously described. The effect of freezing temperature on freezing rate was determined by exposing snails which had an average total body weight and range of 1.69 g (±0.06) to a series of temperatures between −7 and −20° C. The freezing rates were calculated as usual. Exposure temperatures between −7 and −20° C were used since *L. littorea* can prevent the formation of tissue ice at temperatures above −7° C, and is not normally exposed to temperatures below −20° C in the field.

Thawing rates. The influence of total body weight, freezing temperature, and the temperature of the thawing sea water on the thawing rates were determined. The influence of total body weight was defined by exposing specimens of *L. littorea* with total body weights between 0.17 and 4.44 g to −10.0° C, and then thawing the snails by placing each into 120 ml of 30‰ sea water at 0° C. Sea water at 0° C was chosen since this was the average sea water temperature during the water months. The effect of freezing temperature on the thawing rate was determined by exposing snails which had an average total body weight and range of 1.59 g (± 0.07) to temperatures between −7 and −20° C, and then thawing each snail in 120 ml of sea water at 0° C. By exposing snails which had an average total body weight and range of 1.57 g (±0.06) to −10.0° C, and then thawing each of them in 120 ml of 30‰ sea water at temperatures between 0 and 25° C, the influence of the temperature of the thawing medium on the thawing rate was also defined. In all cases, the thawing rates were calculated as previously described.

The influences of total body weight and temperature on the freezing and thawing rates of *L. littorea* were further defined by fitting the non-linear relationships to hyperbolic and exponential curves. The procedure involved transforming the data for each curve into a linear form, calculating a least squares linear regression line, and using these data to define the variables of the non-linear equation (see Sokal and Rohlf, 1969). The goodness of fit was estimated by calculating a correlation coefficient (r) for each line.

Determination of freezing tolerances

To determine the freezing tolerance for any group of snails, the group was subdivided into 4 to 8 subgroups (10 animals/subgroup), and each subgroup was exposed for a defined length of time either to the same freezing temperature, or to a set of different freezing tempratures. Following these low temperature exposures, the animals from each subgroup were removed from the chamber, and immediately placed into 120 ml of 30‰ sea water at a temperature between 0 and 25° C for 15 min to thaw. The thawed snails were placed into fingerbowls containing 200 ml of 30‰ sea water at room temperature (20° C), and were allowed to recover for a defined period of time. On the bottom of each fingerbowl were ten areas marked with an "X". One snail was placed foot down directly over each area. If a snail was not able to crawl the distance necessary to uncover the center of the "X" within a defined recovery time, it was considered dead. Following the recovery period for each subgroup, the percentage of mortality was determined, and differences in freezing tolerances among groups of snails examined by the following methods. For groups of snails exposed to the same freezing temperature, statistical differences among groups were calculated by either a one-way or two-way analysis of variance (ANOVA) and the Student Newman Keuls test (SNK). Statistical differences in freezing tolerance among groups of snails frozen to different temperatures were determined by calculating LD50 values (*i.e.* temperatures at which 50% of the snails died). An LD50 temperature and its 95% confidence limit were calculated according to the statistical procedure of Bliss (1938).

Physical factors influencing freezing tolerance

Freezing and thawing rates. The effect of freezing rate on the freezing tolerance of *L. littorea* was determined by freezing groups of snails which had an average total body weight and range of 1.36 g ± 0.08 to −12.0° C at different rates. A slow freezing rate of 0.14° C/min (±0.03) was obtained by wrapping each snail in a ¼-inch-thick layer of polystyrene, and then exposing the snails to the freezing temperature as previously described. The intermediate rate of 0.38° C/min (±0.10) was accomplished by exposing snails to air at −12.0° C, while the fastest rate of 1.05° C/min (±0.12) was obtained by wrapping each snail fluid tight in aluminum foil, and then exposing the snails to an antifreeze solution (50% (v/v) water: ethylene glycol) at −12.0° C. The freezing rates between 0.14 and 1.05° C/min were examined because they represent the range of freezing rates which the various sizes of specimens of *L. littorea* experience when exposed to air at temperatures between −7 and −20° C (see Results).

Specimens of *L. littorea* frozen to −12.0° C were thawed in 120 ml of 30‰ sea water at 0 and 25° C to achieve thawing rates of 1.7° C/min and 24.5° C/min, respectively. The snails wrapped in aluminum foil, and those with no wrappings were immediately transferred to the thawing medium. The snails wrapped in polystyrene were first unwrapped at −12.0° C, and then transferred to the thawing medium. Thawing rates between 2.7 and 24.5° C/min were chosen since these rates are characteristic of the various sizes of snails frozen to temperatures between −7 and −20° C and thawed in 0° C sea water (see Results).

Following a 15-min thawing period, each group of snails frozen and thawed at a particular rate was allowed to recover for 24 hr, and the percentages of mortality determined. The rates of freezing and thawing were measured and calculated as described previously.

Body weight. The range of total body weights was subdivided into four classes: 0.03 to 0.28, 0.29 to 1.04, 1.05 to 2.65, and 2.66 to 5.50 g. These weight classes correspond to the size classes of 5.0 to 10.0, 10.1 to 15.0, 15.1 to 20.0 and 20.1 to 25.0 mm, respectively. Snail size was measured as the linear distance between the tip of the shell apex and the base of the shell aperture. Subgroups from each class were exposed to a series of freezing temperatures for 6 hr. The snails were thawed and allowed to recover for 24 hr. The LD50 values were calculated for each weight class as previously described.

LD50 values were also determined for the weight classes 0.03 to 0.28 and 20.1 to 25.0 g frozen at the same rate. To freeze specimens of *L. littorea* from different weight classes at the same rate, the following procedure was used. A hole was drilled through the shell of each snail to expose the visceral tissue. Five snails from each weight class were then placed into one of two 50-ml polyethylene test tubes, and covered with 20 ml of 30‰ sea water. Each test tube was then partially submerged in an antifreeze solution at −5° C. When thermal equilibrium was attained, the supercooled sea water was frozen by innoculating with an ice crystal. This procedure resulted in a mean freezing rate of 0.014° C/min with a range from 0.010 to 0.016° C/min. The temperature of the frozen sea water and snails was then lowered from the non-lethal temperature of −5° C to a predetermined temperature at a mean rate of 0.084° C/min with a range from 0.057 to 0.098° C/min. The snails were held at this temperature for 30 min, and then thawed by placing each test tube in 200 ml of sea water at 0° C for 25 min. The snails were allowed to recover as usual, and LD50 values for each class size were calculated as previously described. The freezing rates were controlled manually with the freezing chamber previously described, and the temperature changes were continuously monitored with a thermocouple probe placed into the sea water surrounding the snails.

Freezing time and temperature. To determine the effects of freezing time and temperature on the freezing tolerance of *L. littorea*, groups of snails with a mean total weight and range of 1.41 g ± 0.1 were exposed to temperatures of −9.1,

−11.1 and −13.0° C for periods ranging from 30 min to 7 days. Freezing time was measured from the time thermal equilibrium was attained (point c, Fig. 1). For each temperature and time interval, 11 snails were frozen. Ten snails were used to define the percentage mortality, and the remaining snail had a thermocouple probe inserted into its visceral tissue to monitor the temperature changes. The freezing, thawing, and recovery conditions were the same as usual.

Recovery time and temperature. The influence of recovery time on freezing tolerance was determined by freezing groups of snails to −8.0, −10.0 and −12.0° C for 1 hr, thawing them as usual, and then allowing them to recover in 30‰ sea water at 20° C for periods ranging from 30 min to 7 days. At specific times during the recovery period, the percentage of mortality was determined as usual.

Groups of snails were frozen to a series of temperatures, and allowed to recover in 30‰ sea water at either 4 or 20° C for periods of 48, 96 and 168 hr, to determine the effects of recovery temperature on freezing tolerance. The snails were held in 30‰ sea water at either 4 or 20° C for 2 to 4 weeks prior to freezing to determine the effects of temperature acclimation on the influence of recovery temperature. Both acclimation and recovery temperatures were maintained by 57-liter (15-gal) temperature controlled aquaria. The percentage of mortality for each recovery group was determined at room temperature (20° C), and LD50 values calculated as usual. To reduce the exposure time of the 4° C recovery snails to 20° C, a more rapid procedure was used to estimate mortality. Following a defined recovery time, the snails were removed from the 4° C tank, and allowed to warm up to 20° C by placing them in air at room temperature of 15 min. The percentage mortality was then checked by noting the response of the foot muscle following a mechanical stimulation with a glass probe. Those snails which could completely retract the foot muscle following a stimulation were considered alive. Those snails which could only partially retract the foot were considered dead since preliminary experiments showed that these snails would not be able to crawl, and would be considered dead by the crawling procedure previously described. The tested snails were then placed back into the recovery tank until the next recovery time. The same procedure was applied to the 20° C recovery snails.

RESULTS

Physical factors influencing freezing and thawing rates

The freezing rates of *L. littorea* were influenced by freezing temperatures and total body weights. Lowering the freezing temperature from −7 to −20° C increased the freezing rates of 1.69 g snails 4-fold from 0.15 to 0.57° C/min. The influence of freezing temperature (X) on freezing rate (Y) could be described by the logarithmic equation $Y = 0.98 \log -0.202X$. The correlation coefficient (r) for this equation was 0.94. The freezing rates (Y) of *L. littorea* also increased with decreasing total body weights (X). This relationship can be defined by the hyperbolic equation $Y = 1/(1.04 - 1.58X)$ $(r = 0.98)$. In the population of *L. littorea* examined, the body weights ranged from 0.03 to 5.50 g. When snails with body weights within this range were exposed to −10° C, freezing rates increased 9-fold from 0.1 to 0.9° C/min.

The thawing rates of *L. littorea* were influenced by freezing and thawing temperatures, and total body weights. Lowering the freezing temperatures (X) from −8 to −20° C increased the thawing rates (Y) 3.8-fold from 1.25 to 4.75° C/min.

TABLE I

Influence of freezing and thawing rates on the survival of specimens of L. littorea *frozen to* −12° C *for 1 hr.*

Freezing rate (°C/min)[1]	Thawing rate (°C/min)[1]	% Mortality
0.14 (0.10–0.16)	2.7 (1.5–5.3)	33.3^{a} (±5.6)[2]
	24.5 (21.0–31.4)	$43.3^{a,c}$ (±6.2)
0.38 (0.28–0.48)	2.7 (1.5–5.3)	35.0^{a} (±5.0)
	24.5 (21.0–31.4)	26.7^{a} (±5.6)
1.05 (0.94–1.18)	2.7 (1.5–5.3)	82.0^{b} (±5.8)
	24.5 (21.0–31.4)	$63.7^{b,c}$ (±3.1)

[1] Each value represents a mean and the range, n = 6.

[2] Error values represents ±1 × standard error of the mean, n = 6.

[a,b,c] Means with an identical superscript are not significantly different ($P > 0.05$) (for statistical procedure see Methods section).

This relationship could be described by the exponential equation $Y = 0.51e^{-0.11X}$ ($r = 0.88$). The influence of body weight (X) on the thawing rates (Y) of specimens of *L. littorea* could be described by the hyperbolic equation $Y = 1/(0.06 + 0.29X)$ ($r = 0.97$). Decreasing the total body weights from 5.50 to 0.03 g increased the thawing rates 24.1-fold from 0.60 to 14.5° C/min. Likewise, increasing the temperature of the sea water (X) in which specimens of *L. littorea* were thawed from 0 to 25° C increased the thawing rate (Y) 10.1-fold from 2.9 to 29.8° C/min. This relationship could be defined by the exponential equation $Y = 2.94e^{0.09X}$ ($r = 0.92$).

Physical factors influencing freezing tolerance

Freezing and thawing rates. The survival of specimens of *L. littorea* frozen to −12° C for 1 hr was dependent on the rate of freezing (Table I). Snails frozen at a rate of 0.14° C/min had the same percentage of mortality as snails frozen at a rate of 0.38° C/min ($P > 0.05$). At a freezing rate of 1.05° C/min, however, a significant increase in freezing mortality occurred ($P < 0.001$). Increasing the thawing rate from 2.7 to 24.5° C/min had no effect on the percentage of mortality of specimens of *L. littorea* at any of the freezing rates ($P > 0.05$).

Since the range of total body weights characteristic of *L. littorea* could increase freezing rates from 0.10 to 0.92° C/min, the influence of body weight on freezing tolerance was examined (Fig. 2). Increases in the body weight of specimens of *L. littorea* caused a significant increase in freezing tolerance. Snails in the weight class 0.03 to 0.28 g had an LD50 temperature of −7.37° C, while snails weighing from 2.65 to 5.50 g had a significantly lower LD50 temperature of −12.29° C ($P < 0.001$). The influence of body weight on the freezing tolerance of specimens of *L. littorea* frozen at a controlled rate of 0.08° C/min was also examined. When frozen at the same rate, the snails from 0.03 to 0.28 g weight class had an LD50 temperature of −16.02° C (±0.94), while snails weighing from 2.65 to 5.50 g had an LD50 temperature of −14.27° C (±1.43). Each LD temperature was calculated from 40 animals, and the error values represent 95% confidence limits. These LD50 temperatures were not significantly different ($P > 0.05$).

Freezing time and temperature. Freezing time and temperature were interdependent factors influencing the freezing tolerance of *L. littorea* (Fig. 3). Increasing the extent of freezing time of specimens of *L. littorea* increased the percentage of mortality at each freezing temperature. Specimens of *L. littorea* frozen to −9.1° C were not injured after 12 hr of exposure. Beyond the 12-hr exposure time, however, the percentage of mortality increased linearly until 50% mortality was attained at 168 hr (7 days). Specimens of *L. littorea* frozen to −11.1° C were not injured within the first 3 hr. However, at 28 hr 50% mortality occurred, and within 120 hr 100% mortality was attained. Snails frozen to −13.0° C were injured within the first 30 min of exposure. The freezing mortality increased to 50% within 4 hr, and 100% mortality was attained after only 36 hr of exposure.

Linear regression analysis showed that decreasing the freezing temperature caused a proportional decrease in the log of the time required to reach 50% mortality ($r = 0.999$). For every 1° C drop in freezing temperature, there was a 2.6-fold decrease in the time required to reach 50% mortality.

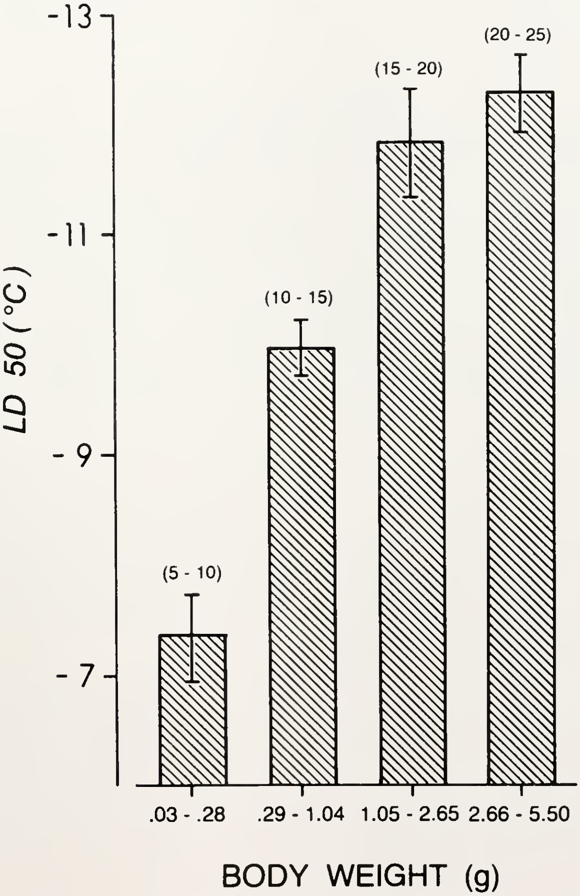

FIGURE 2. Influence of body weight on the freezing mortality of *L. littorea*. The error bars represent 95% confidence limits. Each confidence limit was calculated from 40 to 60 animals. The values above the error bars are the size classes measured in millimeters associated with each weight class.

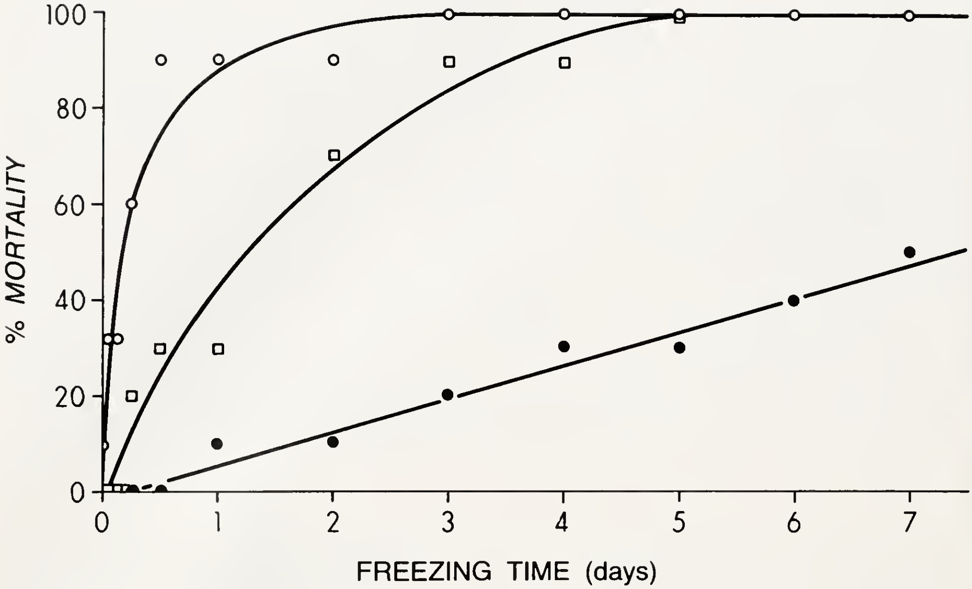

FIGURE 3. Influence of freezing time and temperature on the mortality of *L. littorea.* Closed circles, open boxes, and open circles represent the freezing temperatures of −9.1, −11.1 and −13.0° C, respectively. Each percent mortality value was calculated from 10 animals.

Recovery time and temperature. During the first 48 hr of recovery at 20° C, the percentages of mortality of the snails continually decreased (Fig. 4). The extent of the recovery was dependent on the freezing temperature. Snails frozen to −8.0° C for 1 hr had only a 10% mortality at 48 hr, while snails frozen to −10.0 and −12.1° C had mortalities of 35 and 60%, respectively. Following the 48 hr of recovery, however, the percentages of mortality began to increase, leveling off between 140 to 160 hr. The rate at which the mortality increased was also dependent on the freezing temperature. A 20% increase in freezing mortality occurred within 24 to 48 hr for the snails frozen to −10.0 and −12.1° C, while it took from 72 to 96 hr for a similar increase to occur in the snails frozen to −8.0° C. Likewise, the extent of the freezing injury that occurred after the first 48 hr of recovery was dependent on the freezing temperature. Specimens of *L. littorea* frozen to −8.0° C had percentages of mortality which leveled off at approximately 40%, while the percentages of mortality of the snails frozen to −10.0 and −12.1° C leveled off at approximately 80 and 90%, respectively.

The recovery of specimens of *L. littorea* following a freezing stress was also influenced by the temperature of the recovery sea water (Table II). The LD50 temperatures of the snails recovered at 4° C ranged from −14.55 to −13.43° C, while the LD50 temperatures of the snails recovered at 20° C ranged from −12.82 to −11.68° C. The greater freezing tolerance of the snails recovered in 4° C sea water occurred following acclimation at either 4 or 20° C. In all cases, the LD50 temperatures rose by approximately 1° C from 48 to 168 hr of recovery.

DISCUSSION

The results of this research indicate that the freezing tolerance of the marine snail *L. littorea* is dependent on the interaction of various physical and temporal

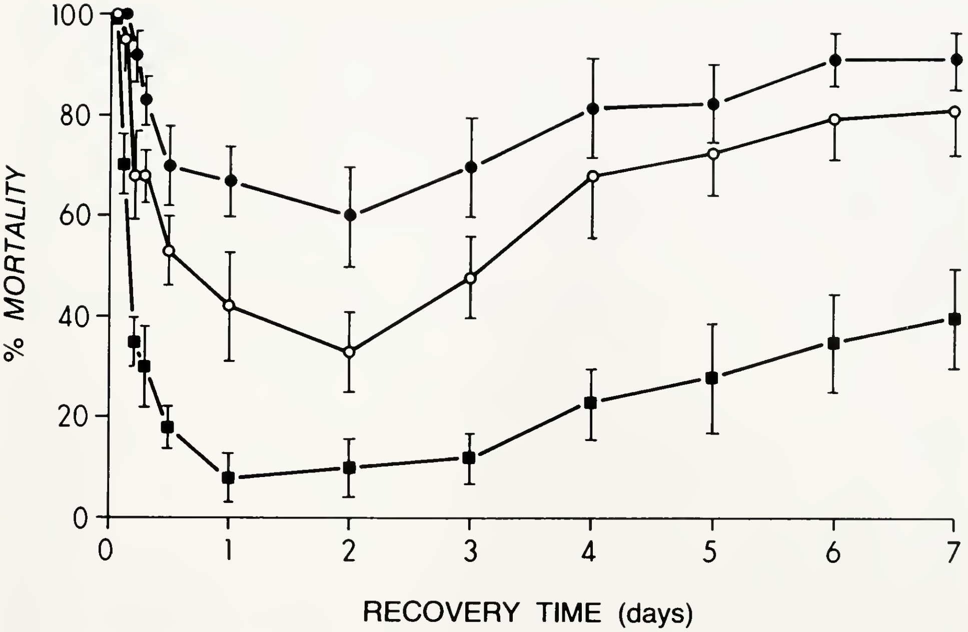

FIGURE 4. Influence of recovery time on the freezing mortality of *L. littorea.* All snails were recovered in 20° C sea water. Closed boxes, open circles, and closed circles represent the freezing temperatures of −8.0, −10.0, and −12.1° C, respectively. All snails were frozen in air for 1 hr, and thawed in 0° C sea water prior to recovery. Each mortaility value was calculated from 60 animals. The error bars represent ±1 × standard error of the mean (n = 6).

factors during and following the freezing stress. The rate at which *L. littorea* froze influenced its freezing tolerance, while changes in thawing rates had no effect. Increasing the freezing rate above 0.4° C/min reduced freezing tolerance.

TABLE II

Influence of recovery temperature on the freezing tolerance of specimens of L. littorea *acclimated at 4 and 20° C.*

Acclimation temperature (°C)	Recovery temperature (°C)	Recovery time (hr)	LD50 values (°C)[1]
4	4	48	−14.55 (±0.94)[2]
		96	−14.55 (±0.94)
		168	−13.77 (±0.51)
	20	48	−12.82 (±0.92)
		96	−12.39 (±0.32)
		168	−11.99 (±0.47)
20	4	48	−14.55 (±0.71)
		96	−14.52 (±1.30)
		168	−13.43 (±0.62)
	20	48	−12.49 (±1.13)
		96	−11.75 (±0.57)
		168	−11.68 (±0.98)

[1] LD50 values were calculated from 40 to 60 animals.
[2] Error values represent 95% confidence limits.

A very similar dependence of freezing tolerance on freezing rate also occurs in arctic insects (Miller, 1978). Since increases in freezing injury at greater freezing rates are due to the formation of intracellular ice in many types of cells (Mazur, 1977), these data suggest that the increase in freezing injury in specimens of *L. littorea* at freezing rates above 0.4° C/min is also due to the mechanical disruption of cellular structures caused by the formation of intracellular ice. Conversely, at freezing rates below 0.4° C/min, it appears that only extracellular ice formation occurs. The formation of extracellular ice has been observed in specimens of *L. littorea,* and injury apparently results from cellular dehydration (Kanwisher, 1955).

Total body weight and freezing temperature are important physical factors influencing the freezing rate of *L. littorea.* The difference in total body weight was the most important factor, and could alter the freezing rate of *L. littorea* by a factor of 9. An examination of the effects of body weight and freezing temperature on freezing rates indicates that the freezing rates in a population of *L. littorea* could range anywhere from 0.06° C/min for the largest snails exposed to −7° C, to 1.60° C/min for the smallest snails exposed to −20° C. Since these values extend above and below 0.4° C/min, it appears that freezing rate is an important factor influencing the freezing tolerance of *L. littorea.* Indeed, the ability of larger specimens of *L. littorea* to tolerate lower freezing temperatures was due solely to differences in freezing rates. The importance of freezing rate is further supported by the finding that small specimens of *L. littorea* tend to disappear from the higher intertidal zones along the Massachusetts coast during the colder winter months (Gendron, 1977).

Changes in temperature and time during a freezing stress also influence the freezing tolerance of *L. littorea.* For every 1° C drop in temperature, the time required for specimens of *L. littorea* to reach 50% mortality was reduced 2.6 times. Thus, although specimens of *L. littorea* could tolerate −8° C for approximately 8 days, they could survive at temperatures of −12 and −13° C for periods of only 6 and 2.3 hr, respectively. Since *L. littorea* inhabits the intertidal zone, it is exposed to freezing temperatures only periodically during aerial exposures at low tides. The extent of these exposures are dependent on vertical distributions in the intertidal zone. For example, snails living below the mean tidal level (MTL) are exposed to air for periods of less than 6 hr at a time. On the other hand, snails inhabiting areas from the MTL up to the mean high water spring tidal level (MHWS) are exposed for periods ranging from 6 hr to 2 weeks at a time. Since reducing the time frozen can lower the lethal freezing temperatures of *L. littorea,* changes in the vertical distributions of this animal may be an important factor influencing its survival during the colder winter months.

The extent of the freezing injury that occurs in specimens of *L. littorea* during a freezing stress will change during the period following the freezing stress. During the first 48 hr after the freezing stress, the extent of the freezing damage is reduced. Thus, some type of repair process is occurring during this time. After these first 48 hr, however, the freezing damage begins to increase, and levels off after approximately one week. Since the ability to crawl was used to estimate the extent of freezing injury, it appears that although the muscle tissue directly associated with crawling was able to initially recover from the freezing stress, other tissues within the organism may have been more severely damaged resulting in a latent, or secondary, injury to the muscle. To determine whether this is the case, the relative freezing tolerances of the various tissues within *L. littorea* will have to be determined.

The injury that resulted from the freezing stress was also influenced by the temperature of the recovery medium. The specimens of *L. littorea* which were placed into 4° C sea water following the freezing stress had less freezing injury than snails placed into 20° C sea water. Acclimation of the snails to either 4 or 20° C prior to the freezing stress had no effect on this response. Thus, the differences in freezing injury were not due to a secondary thermal stress resulting from differences in the temperatures existing before and after freezing. Although this response to recovery temperature has also been observed in plants (Levitt, 1972) and mammalian cells (McGann *et al.*, 1975), the mechanism responsible for this effect remains unknown.

SUMMARY

The ability of *Littorina littorea* to tolerate freezing and thawing was influenced by body weight, temperature and time. The freezing tolerance of *L. littorea* was dependent on freezing rate, with values above 0.4° C/min reducing freezing tolerance. Increases in body weight produced greater freezing tolerances by reducing the freezing rate. The length of time and the temperature to which specimens of *L. littorea* were frozen also influenced freezing tolerance. These factors were interdependent. Over the temperature range of -9 to $-13°$ C, every 1° C drop in temperature produced a 2.6-fold decrease in the time required to produce freezing injury. The freezing tolerance of *L. littorea* was independent of thawing rates.

Recovery time and the temperature of the recovery medium also influenced the freezing tolerance of *L. littorea*. The freezing injury that occurred during freezing and thawing was reduced during the first 48 hr of recovery. After the first 48 hr, however, the injury began to increase, leveling off after approximately 1 week. Further, the snails recovered in 4° C sea water had a lower mortality than those recovered in 20° sea water.

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INFLUENCE OF COPPER ON THE CLAM *PROTOTHACA STAMINEA*: EFFECTS ON GILLS AND OCCURRENCE OF COPPER-BINDING PROTEINS[1]

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Copper contamination of the coastal marine environment has been a subject of increasing concern due to increases in anthropogenic inputs (Helz, Huggett, and Hill, 1975) and the high toxicity of copper to marine organisms (Eisler and Wapman, 1975). Despite the fact that copper is known to be an essential trace element for biological processes (Adelstein and Vallee, 1962), extremely low concentrations of copper have been shown to be lethal to several marine species. The natural levels of copper in sea water could be toxic to some species were it not for the presence of substances in sea water which complex with the metal and render it less available for uptake (Steeman-Nielsen and Wium-Anderson, 1970; Sunda and Guillard, 1976; Gnassia-Barelli, Romeo, Laumond, and Pesando, 1979; Engel and Sunda, 1979). The ionic form of copper appears to be the toxic form of copper to marine organisms (Sunda and Guillard, 1976; Engel and Sunda, 1979).

The dual nature of copper as both essential trace element and a potential toxin at extremely low levels of exposure would demand that organisms strictly regulate copper at internal levels suitable for metabolic requirements. Copper is normally present at relatively high levels in tissues of marine animals (> 1 μg/g) (Goldberg, Bowen, Farrington, Harvey, Martin, Parker, Risebrough, Robertson, Schneider and Gamble, 1978) when compared to natural concentrations in sea water (< 1 μg/l) (Batley and Gardner, 1978) and, as has been shown with zinc (Wolfe, 1970; Coombs, 1972; 1974), is probably present at levels in excess of cofactor requirements for enzymes. Copper is known to occur intracellularly as granules and within membrane-bound vesicles resembling lysosomes (Coombs and George, 1978) and bound to soluble, low molecular weight, metallothionein-like proteins (Howard and Nickless, 1977a) or to smaller organic molecules (Coombs, 1974; Howard and Nickless, 1977b; 1978). Those compartments may represent intracellular storage depots for excess copper and serve as protective or detoxification mechanisms (Coombs and George, 1978; Cherian and Goyer, 1978).

The existence of metallothionein-like, low molecular weight, metal-binding proteins has been established for several species of marine invertebrates (Casterline and Yip, 1975; Noel-Lambot, 1976; Howard and Nickless, 1977a; Brown, Bawden, Chatel, and Parsons, 1977; Brown and Parsons, 1978; Talbot and Magee, 1978; Jennings, Rainbow and Scott, 1979; Olafson, Sim, and Boto, 1979; Overnell and Trewhella, 1979; Ridlington and Fowler, 1979) including several molluscan and crustacean species and zooplankton of unknown species composition. Positive identification of the protein as metallothionein has, thus far, been made on the brachyuran crab species *Scylla serrata* (Olafson, Sim and Boto, 1979) and *Cancer pagurus* (Overnell and Trewhella, 1979). For the most part, the studies have emphasized the identification of a cadmium-binding protein (Casterline and

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Yip, 1975; Noel-Lambot, 1976; Talbot and Magee, 1978; Jennings, Rainbow and Scott, 1979; Olafson, Sim and Boto, 1979; Ridlington and Fowler, 1979) although a copper-binding protein has been reported in limpets collected from a copper-contaminated environment (Howard and Nickless, 1977a) and in the gills of the mussel *Mytilus edulis* exposed to copper (Viarengo, Pertica, Mancinelli, Palmero and Drunesu, 1980). The association of other metals, particularly copper and zinc, with metal-binding proteins has usually been made with cadmium-exposed animals. Thus, although it is now well known that metallothioneins in mammals can be induced by a number of trace metals (Suzuki and Yoshikawa, 1976) and is especially important in the binding of cadmium, zinc, copper and mercury (Kojima and Kagi, 1978), parallel information for the invertebrate proteins is not available.

In the present study, clams (*Protothaca staminea*) were exposed for 30 days to a range of copper concentrations from background levels in sea water (= 0.35 μg/l total Cu) to 82 μg/l; the latter resulting in 97% mortality within 30 days. Gills were the primary organ for the concentration of copper and may have been a target for the toxic action of the metal. Examination of the gills for sodium and potassium content and acid phosphatase activity indicated effects associated with both lethal and sublethal alterations. Gel chromatographic analysis of the cytoplasmic fraction of clam tissues provided evidence for the existence of a low molecular weight copper-binding protein and the possibility of a separate protein similar to metallothionein with respect to molecular weight and its ability to bind copper, zinc and cadmium.

Materials and Methods

Clams were collected from the intertidal zone of Sequim Bay, Washington, during May, 1978. Clams which were 5.2- to 5.8-cm total length were selected for the study and held in tanks containing flowing sea water and Crystal Amber #8 Aqua Monterey beach sand (Monterey Sand Co., Monterey, Calif.) for 11 to 15 days before use in the experiment. Seawater temperatures during holding varied from 12.2 to 12.5° C and salinity was 31‰.

Experimental procedures

Clams were exposed to measured copper concentrations of control (= 0.35), 7, 18, 39 and 82 μg/l total copper for 30 days, 36 clams per treatment. Copper for exposures was added as $CuSO_4$ in deionized water. Exposures were conducted in 81 liter fiberglass tanks containing 9 cm of Crystal Amber #8 Aqua Monterey beach sand for substrate and continuous flows of sea water and added copper. Sea water was delivered at 1.5 liter per minute (min) to the tanks via a polyvinyl chloride (PVC) manifold connected to a constant head tank and the copper via a peristaltic pump from one of two stock concentrations at 3 or 6 ml/min. The sea water and copper solutions mixed in funnels above the tanks before delivery of the mixture to the tanks by a PVC diffuser system.

Mortalities were monitored daily, and dead clams were discarded. Mean seawater temperature, salinity, pH, and dissolved oxygen levels in the exposure tanks were 12.3° C, 31.2‰, pH 8.1 and 8.3 mg/l dissolved oxygen, respectively, and did not vary among tanks. At the end of the 30-day exposure, surviving clams were prepared for copper analysis of dissected organs; sodium, potassium, calcium and magnesium concentrations and acid phosphatase activity of gills; or frozen at −65° C for later analysis of copper-binding proteins.

Analytical procedures

Copper in exposure sea water was measured by anodic stripping voltammetry (Young, Gurtisen, Apts and Crecelius, 1979) at pH 8 for ionic and/or weakly complexed species and at pH 2 for total copper. Temperature, salinity, pH and dissolved oxygen were analyzed as previously described (Roesijadi, Jacobsen, Bridge and Crecelius, 1979).

Concentrations of copper, sodium, potassium, calcium and magnesium in clam tissue were determined with an Instrumentations Laboratories (IL) 251 atomic absorption spectrophotometer. For copper measurements, clams were dissected into gills, kidney, muscle (= abductor muscles, foot and mantle) and the remaining visceral mass which contained the digestive gland, gonads and other organs. Other ions were measured in the gills only. Gills were rinsed in isosmotic sucrose to remove adhering sea water and hemolymph, blotted dry on tissue paper, then weighed. All tissues were dried for 5 days at 60° C; digested in hot, concentrated, nitric acid; taken to dryness; then resolubilized in 0.4 N nitric acid prior to analysis. Copper concentrations were expressed as micrograms Cu per gram dry weight. Concentrations of other ions in gills were expressed as millimoles ion per kg tissue water (tissue water = wet weight − dry weight).

The activity of the lysosomal marker enzyme acid phosphatase (de Duve, Pressman, Gianetto, Wattiaux and Appelmans, 1955) was determined in freshly excised gills by measuring the rate of hydrolysis of p-nitrophenyl phosphate at pH 5 (Barrett, 1972). Gills were homogenized in 0.75 M sucrose with a Dounce-type homogenizer. Homogenates were centrifuged at 1,000 *g* for 10 min at 4° C. The resultant supernatants were subjected to thrice freezing in liquid nitrogen and thawing under cold tap water, then centrifuged again at 35,000 *g* for 15 min at 4° C. Final supernatants were pre-incubated at 10° C for 30 min, then analyzed for acid phosphatase activity. Incubation during the assay was for 1 hr at 10° C. Proteins in enzyme mixtures were measured by the method of Lowry, Risebrough, Farr and Randall (1951), with bovine serum albumin as standard. Enzyme activities are reported as the appearance of micromoles (μM) p-nitrophenol per mg protein/hr.

Gel chromatography of soluble tissue extracts was conducted with a 1.6 × 85 cm column of Sephadex G-75 fine gel (Pharmacia Fine Chemicals, Piscataway, N. J.) and 0.1 N NH_4HCO_3 as eluting buffer. Flow rate was 0.3 ml/min. The column was set in a 4° C incubator. Clams were dissected as described above, and pooled tissues of five clams were homogenized on ice in two volumes of 0.75 M sucrose containing 1% 2-mercaptoethanol. This procedure has been found to minimize aggregation of copper-binding proteins and to maximize their extraction (Ryden and Deutsch, 1978). Homogenates were centrifuged at 35,000 *g* for 15 min at 4° C. Supernatants were removed, heated to 70° C and held at that temperature for 1 min, then centrifuged again as above (Cherian, 1974). The final supernatants were frozen at −65° C until chromatographed. Chromatographic effluents were collected as 5 ml fractions. Each fraction was analyzed for absorbance at 254 and 280 nm, for protein by the Coomassie brilliant blue G-250 dye-binding method (Bio-Rad Protein Assay, Bio-Rad Laboratories, Richmond, Calif.), and for copper, zinc, and cadmium by aspirating directly into the flame of

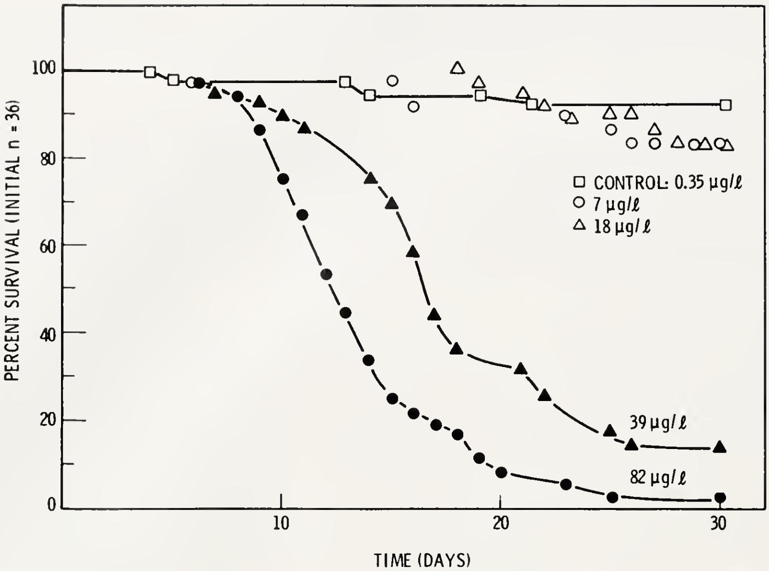

FIGURE 1. Percent survival of specimens of *Protothaca staminea* during 30-day exposure to control, 7, 18, 39, or 82 µg/l total copper in sea water.

an IL 251 atomic absorption spectrophotometer. Bovine serum albumin was used as the protein standard. Deuterium background correction was applied during analyses for metals in column fractions.

Calibration standards for the chromatographic column consisted of blue dextran (void volume), albumin (MW = 67,000 daltons), ovalbumin (43,000), chymotrypsinogen (25,000) and ribonuclease A (13,000) (Pharmacia Fine Chemicals).

All chemicals used in the study were reagent grade or better. Water was deionized using a system of single anion-cation exchange columns and mixed bed anion-cation exchange columns. Glassware was soaked in 50% HNO_3 followed by 50% HCl as part of routine cleaning procedures.

RESULTS

With the exception of the control situation, copper in the various experimental treatments was present mainly as ionic or weakly complexed chemical species. More strongly complexed forms of copper were present at levels of 1 to 3 µg/l in tanks with added copper. In the control tank, virtually all the copper was believed to be in the strongly complexed form since ionic/weakly complexed forms were not detectable. The copper concentration for control sea water of 0.35 µg/l was consistent with concentrations reported for Sequim Bay sea water (Young, Gurtisen, Apts and Crecelius, 1979) and other natural marine waters (Batley and Gardner, 1978). Copper concentrations as high as those used for exposures (7 to 82 µg/l) have been reported for extremely contaminated coastal waters (Wildhauer, Matte and Tucker, 1978).

Survival of clams during the 30-day exposure (Fig. 1) was high in controls (= 97%) and slightly reduced at 7 and 18 µg/l copper (= 86% and 83% survival, respectively). Exposures of 39 and 82 µg/l proved to be extremely stressful with only five of 36 clams surviving at 39 µg/l (14% survival) and one of 36 at

82 μg/l (3% survival). Time to 50% mortality was 17 and 12 days, respectively, at those higher copper concentrations.

Copper analyses of clam tissues indicated that copper-exposed clams possessed higher concentrations of copper in all organs when compared to control clams. However, gills were the only organs to exhibit a continued increase in the tissue copper concentration with a corresponding increase in the seawater copper concentration (Fig. 2). The relationship between gill copper concentration and seawater copper concentration up to 39 μg/l was essentially linear. (See Table I for copper concentrations in the other organs.)

Gills were further examined for the concentrations of the inorganic ions calcium, magnesium, sodium, and potassium as measures of cellular integrity since concentrations of those ions are believed to be under regulatory control (Prosser, 1973). Calcium and magnesium were present at concentrations of 13.1 ± 1.7 (s.e.) and 30.9 ± 1.5 millimoles/kg tissue water, respectively, in control clams. No differences between control and exposed groups were observed in the levels of those two ions. With gill sodium and potassium, however, differences were observed at an exposure concentration (39 μg/l) which was lethal (14% survival) but not at lower exposure concentrations at which survival was relatively high (Fig. 3). Ion concentrations are plotted against gill copper concentrations to show the relationship between those two parameters. At the 39 μg/l copper exposure, the increases in sodium and decreases in potassium indicated a disruption in the regulation of those ions.

At the lower exposure concentrations of 7 and 18 μg/l at which effects on sodium and potassium levels of gills were not observed, substantial increases were observed in the activity of acid phosphatase (Fig. 4). Increases in enzyme activity were linear with respect to gill copper concentration, suggesting a sublethal dose-response relationship; perhaps a cytotoxic action of copper due to

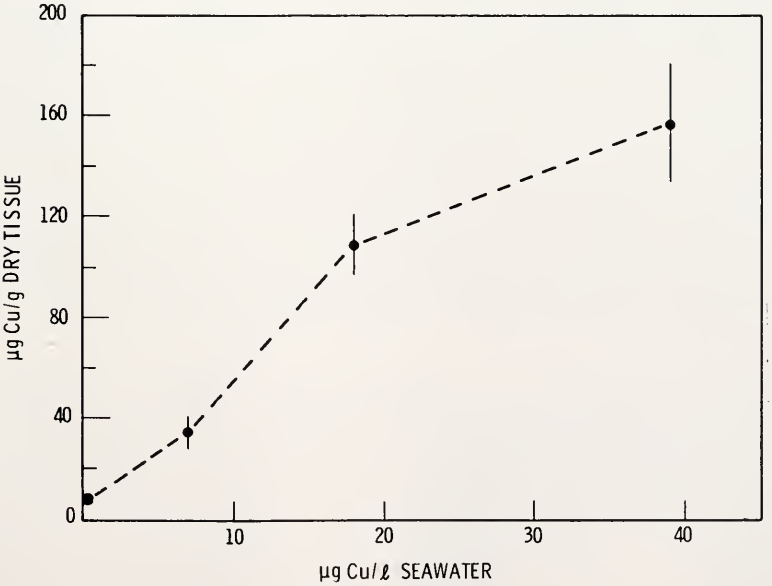

FIGURE 2. Copper content of gills following 30-day exposure to control, 7, 18, or 39 μg/l copper in sea water. Mean ± 1 standard error.

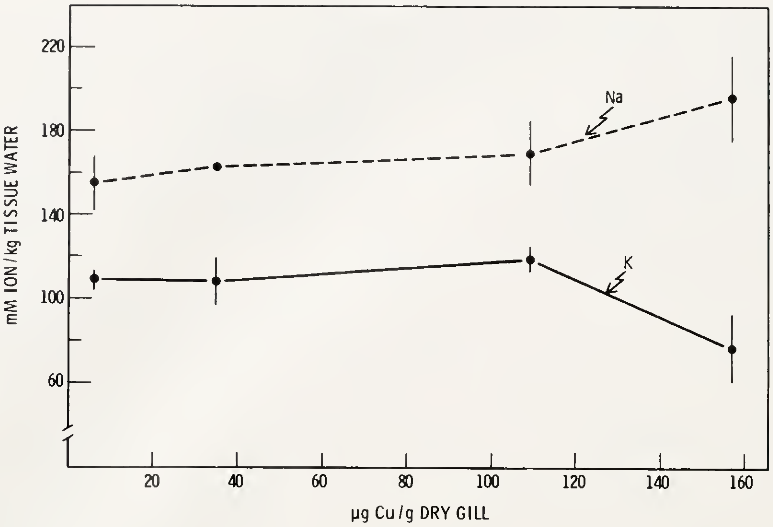

FIGURE 3. Sodium and potassium content of gills following 30-day exposure to control, 7, 18, or 39 μg/l total copper in sea water. Gill ion concentrations are plotted against the gill copper cencentrations of Figure 2. Mean ± 1 standard error.

increased lysosomal activity. Mortalities precluded measurements for clams at higher exposures of copper.

Copper concentrations in other organs (Table I) were not directly correlated with the exposure copper concentration in the manner described above for gills. Muscle and viscera copper concentrations reached an apparent maximum at the 7μg/l exposure. Kidneys exhibited an increase to 111 μg copper/g dry weight at the 7 μg/l exposure and a decline to 27.2 μg/g at the 18 μg/l exposure. Although the significance of the high kidney copper concentration at 7 μg/l is not understood, the possibility of copper contamination can be discounted as the analyses have been repeated on separate kidney samples with similar results. Whole animal copper concentrations exhibited a pattern similar to that described for muscle and viscera. The importance of examining individual tissues or organs for metals content is indicated by the results, since patterns present in small organs such as gills or kidneys were not reflected in whole animal preparations.

Gel chromatography of the soluble extracts of clam tissues from the control, 7 and 18 μg/l treatments indicated the presence of low molecular weight, metal-binding proteins in copper-exposed, as well as control, clams. However, it appears

TABLE I

Concentrations of copper (Mean ± 1 standard error μg/g dry weight, n = 5) in various organs of Protothaca staminea following exposure for 30 days to total copper levels indicated below.

Body component	Control	7 μg/l	18 μg/l
Gill	7.5 ± 0.5	35.3 ± 7.1	109.5 ± 12.5
Muscle	4.3 ± 0.4	34.7 ± 7.9	35.0 ± 6.6
Viscera	9.3 ± 1.7	57.7 ± 17.0	54.5 ± 18.9
Kidney	5.3 ± 0.3	111.1 ± 36.0	27.2 ± 13.6
Whole Animal	5.6 ± 0.4	47.7 ± 11.7	46.7 ± 11.0

that the protein predominant in copper-binding may not be the same in different organs. To illustrate, chromatograms for the 18 μg/l copper-exposed clams are presented in Figure 5. Measurements for 280 and 254 nm absorbance (only 280 nm trace shown here since absorbance at 280 and 254 nm exhibited similar patterns) indicated strongly absorbing material at the void volume (Vo) (66.5 ml; > 70,000 daltons) and in the low molecular weight pool (> 150 ml; < 3,000 daltons) and a lack of such material in the region of the low molecular weight, metal-binding proteins (100 to 150 ml elution volume [Ve]). Analysis of proteins using the Coomassie brilliant blue dye-binding method verified the proteinaceous nature of the substances in the region of the low molecular weight proteins, although no distinct protein peaks were observed in the latter. In muscle tissue an unidentified protein peak of about 22,000 daltons was also observed. In gill and muscle tissues, low molecular weight copper- and zinc-binding protein peaks did not coincide, suggesting the presence of two separate proteins in the binding of those metals. The copper peak was estimated as having a molecular weight of approximately 14,000 daltons and the zinc peak with 10,500 daltons (determined from linear regression of Ve/Vo versus $\log_{10}$ molecular weight). In viscera and kidney, copper and zinc peaks coincided on the 10,500 daltons protein (Fig. 5). High amounts of cadmium were also associated with the kidney protein. The behavior of the smaller protein with respect to its apparent molecular weight and its ability to bind copper, zinc, and cadmium is similar to that of metallothionein. The distribution of copper on separate low molecular weight proteins in the different organs was also observed in control clams and those exposed to 7 μg/l

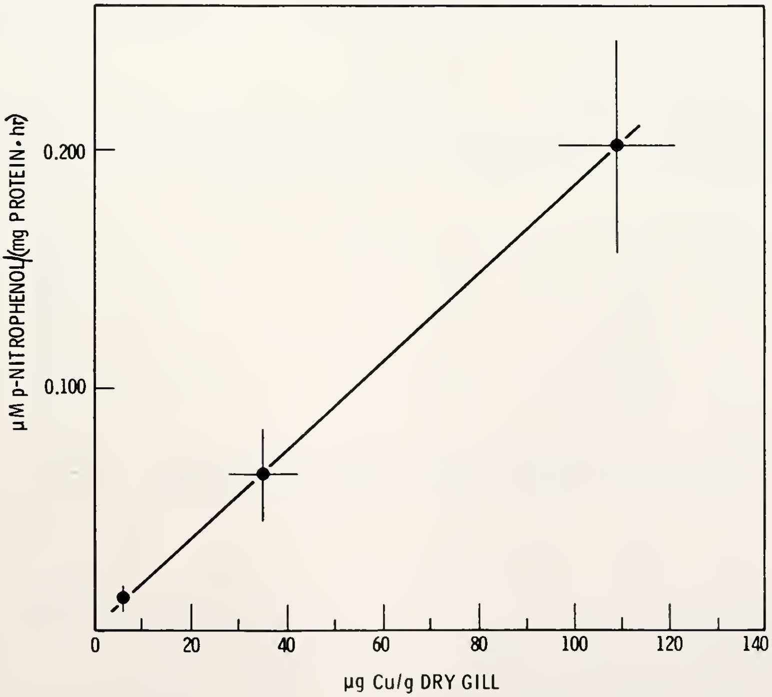

FIGURE 4. Acid phosphatase activity of gills following 30-day exposure to control, 7, or 18 μg/l total copper in sea water. Enzyme activities are plotted against the gill copper concentrations of Figure 2. Mean ± 1 standard error.

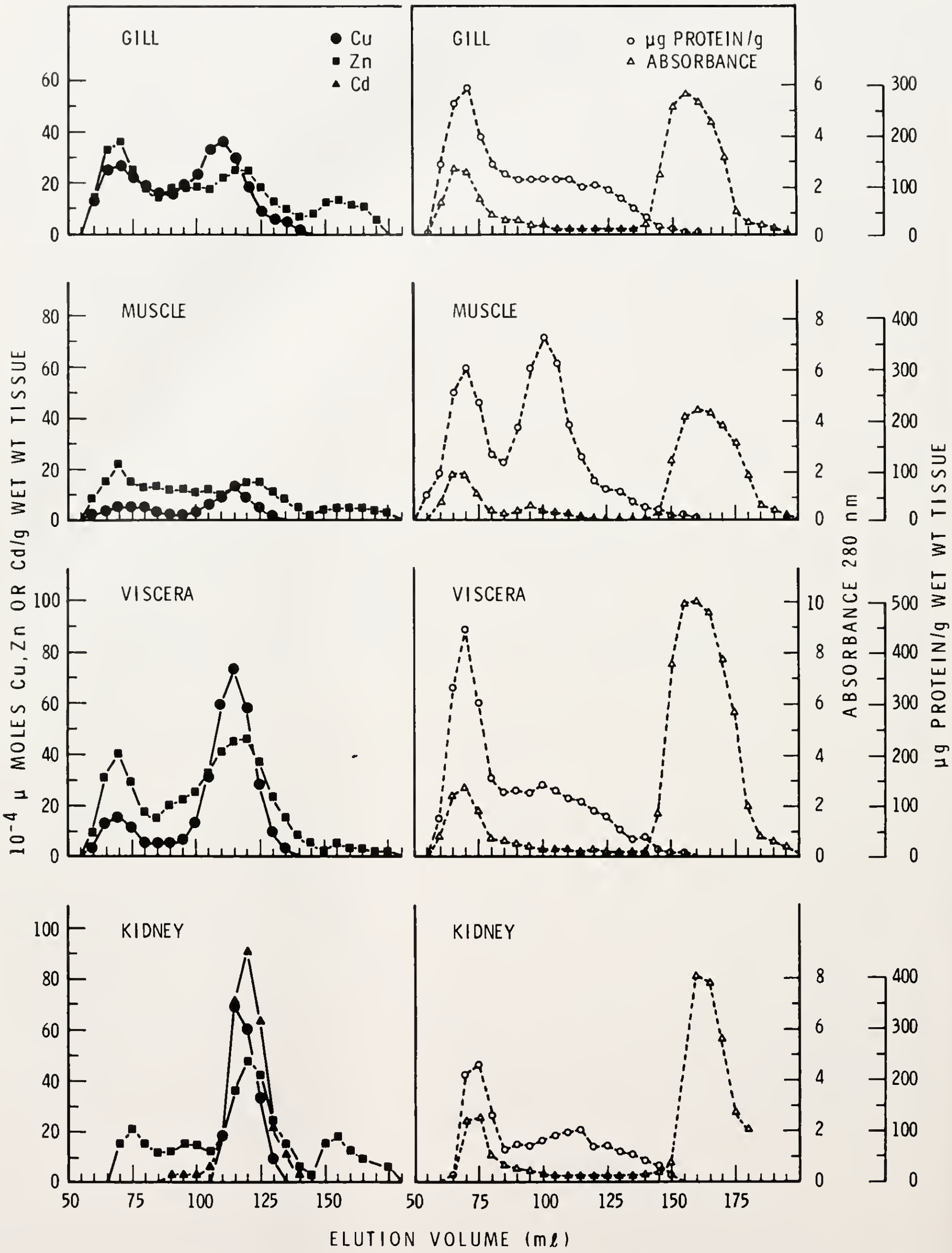

Figure 5. Sephadex G-75 gel chromatography of tissue extracts of 18 μg/l copper-exposed clams. Values for each fraction are expressed on basis of wet weight of tissue by the following correction: metal concentration or absorbance of fraction × volume of fraction/volume chromatographed × (wet weight + buffer volume)/wet weight.

copper. The relatively large quantities of copper associated with the high molecular weight proteins which occurred at the void volumes in Figure 5 were due to the increased binding of copper as a result of copper exposure. Copper was not detected in the low molecular weight pool components indicating that there was little or no free copper or copper bound to substances less than 3,000 molecular weight.

Values for copper, zinc and cadmium associated with the various tissues for all copper treatments are summarized in Table II. To calculate those values, metal concentrations for the three fractions which contained the highest amounts from a single peak were summed and expressed on the basis of wet weight.

In control clams significant quantities of metals were associated with low molecular weight proteins, suggesting a normal role for those proteins in metals' binding. Viscera and kidney, in particular, of controls possessed high levels of such protein-bound metals. Additionally, it appears that the metal-binding protein of kidneys is an important reservoir for excess cadmium, which composed 40% of the total molar quantity of the soluble copper, zinc and cadmium of kidneys. The occurrence of relatively high levels of zinc associated with high molecular weight proteins suggested the presence of extractable zinc metalloproteins and, possibly, relatively stable metal-protein complexes. Little or no copper or cadmium was detected in the high molecular weight fractions of controls.

Exposure to copper led to increases of copper on the low molecular weight proteins, as well as increases on high molecular weight proteins (Table II). It is suspected that binding of copper to the former substances is a specific response to excess intracellular copper, possibly for sequestration and detoxification, while binding to the latter may be a non-specific response and related to toxic processes.

Decreases of zinc on the low molecular weight proteins which accompanied the increases of copper in all tissues suggested metal interactions, possibly a competition for binding sites. However, the increase in copper cannot be solely

TABLE II

Cu, Zn, Cd, and Σ Cu, Zn, Cd concentrations on high and low molecular weight (MW) protein peaks.[1]

Organ	Treatment	High MW Proteins				Low MW Proteins[2]			
		Cu	Zn	Cd	Σ	Cu	Zn	Cd	Σ
Gill	Control	4	65	n.d.[3]	69	24	99	6	129
	7 μg/l	87	78	n.d.	165	141	72	n.d.	213
	18 μg/l	75	93	n.d.	168	99	72	n.d.	171
Muscle	Control	n.d.	69	n.d.	69	3	63	n.d.	66
	7 μg/l	54	54	n.d.	108	36	45	n.d.	81
	18 μg/l	12	51	n.d.	63	30	54	n.d.	84
Viscera	Control	14	141	2	157	105	294	10	409
	7 μg/l	129	141	3	273	360	129	3	492
	18 μg/l	39	99	n.d.	138	189	132	n.d.	321
Kidney	Control	n.d.	39	n.d.	39	21	159	147	327
	7 μg/l	53	65	12	130	552	69	53	674
	18 μg/l	5	51	n.d.	56	162	126	222	510

[1] Concentration unit is 10^{-4} micromoles metal/g wet weight.

[2] Copper on low molecular weight proteins of gill and muscle was associated with the 14,000 daltons "copper-binding protein." Copper on low molecular weight proteins of viscera and kidney and zinc and cadmium on low molecular weight proteins of all organs were associated with the 10,500 daltons metallothionein-like protein.

[3] n.d. = not detectable.

attributed to displacement of zinc or other metals from the low molecular weight proteins since total metal levels on the proteins increased following copper exposure. In kidney, the increase in copper on the metallothionein-like proteins at the 7 μg/l exposure was accompanied by a decrease in cadmium, as well as zinc. At the 18 μg/l exposure, zinc had decreased on that protein, while both copper and cadmium concentrations increased. Interactions between copper and other trace metals on low molecular weight proteins are complex, and the details are not presently understood.

The soluble proteins from tissues of clams exposed to 18 μg/l copper contained total copper levels lower than those from the 7 μg/l treatment (Table II). The decline in the soluble copper at 18 μg/l was associated with an increased binding of copper to the pelleted fraction of tissue homogenates. This shift in the subcellular distribution of copper may be related to toxic processes. It was shown earlier that 18 μg/l copper in sea water approached the lethal concentration for a 30-day exposure.

Discussion

The toxicity of copper to molluscs has been demonstrated in numerous studies (Eisler and Wapman, 1975). The sensitivity of *Protothaca staminea* to copper exposure (time to 50% mortality: 12 days at 82 μg/l) was not unlike that reported for the mussel *Mytilus edulis* (time to 50% mortality: 10 days at 90 μg/l) by Davenport and Manley (1978). All organs of *P. staminea* accumulated copper during copper exposure. However, gills were found to be the primary organ for the concentration of copper and exhibited a linear increase in copper with increased exposure concentration. This finding is consistent with the accepted role of the bivalve gill as one of the major organs for the uptake and exchange of solutes with the external media. Tissue copper concentrations in clams exposed to 18 μg/l copper for 30 days may have approached maximum tolerable tissue concentrations since it is apparent that higher exposures would have resulted in certain death for most of the clams.

The possibility that the gills, which were the primary organ for the concentration of copper, were also adversely affected by copper exposure, was demonstrated by measurements for sodium, potassium, and acid phosphatase activity. At the relatively high exposure concentration of 39 μg/l at which mortalities were high, sodium and potassium concentrations of gills were altered in a manner suggestive of effects on membrane regulatory processes involved in the expulsion of sodium and concentration of potassium. At the lower exposure concentrations, such processes appeared to remain unaffected since no changes in ionic concentrations were observed. Disruption of cellular ion regulation as a result of heavy metal exposure may be related to inhibition of sodium-potassium adenosine triphosphatase (Na-K ATPase) activity (Renfro, Schmidt-Nielsen, Miller, Benos and Allen, 1974; Schmidt-Nielsen, 1974). Attempts to use Na-K ATPase activity of gills as an assay in this experiment were not successful due to the low and variable enzyme activities.

The increase in the activity of acid phosphatase of gills at sublethal exposures to copper may have resulted from the cytotoxicity of copper, possibly through increased turnover of organelles and, hence, increased lysosomal activity (Ericsson, 1969). Lysosomes also directly interact with toxic trace metals in the detoxification of the metal or as the target of toxicity (Sternlieb and Goldfischer, 1976). The ability of lysosomes to sequester and store toxic metals is well known (Sternlieb and Goldfischer, 1976; Coombs and George, 1978), as is the labilization of lysosomes and their release of hydrolytic enzymes following excess exposure to various toxic metals, including copper (Sternlieb and Goldfischer, 1976; Moore and Stebbing, 1976).

It has been reported for mammalian liver that copper can occur on at least two low molecular weight proteins, copper chelatin or metallothionein, depending on whether induction of the proteins initially occurred as a result of exposure to copper or to another trace metal such as cadmium (Winge, Premakumar, Wiley and Rajagopalan, 1975). Copper-chelatin is reported to be specific for copper (Winge, Premakumar, Wiley and Rajogopalan, 1975; Premakumar, Winge, Wiley and Rajogopalan, 1975a; Premakumar, Winge, Wiley and Rajapogalan 1975b; Rajagopalan, Winge and Premakumar, 1976; Day, Coles and Brady, 1978) and has been identified from several eucaryotic sources (Premakumar, Winge, Wiley and Rajagopalan, 1975b). Copper on metallothionein is usually associated with zinc or cadmium (Winge, Premakumar, Wiley and Rajagopalan, 1975, Bremner and Marshall, 1974). In the present study, copper was shown to occur on two distinct peaks, one of which possessed metal-binding properties similar to metallothionein and the other to copper-chelatin. The metallothionein-like protein described here was bound to copper, zinc, and cadmium and estimated as 10,500 daltons, a molecular weight not dissimilar from metallothionein when estimated by Sephadex gel chromatography (Nordberg, 1978). The "copper-binding protein" was estimated as 14,000 daltons which is approximately twice larger than the 7,600 estimate for copper-chelatin (Winge, Premakumar, Wiley and Rajagopalan, 1975). While it is possible that the dimer of the "copper-binding protein" was detected in the present study, it should be noted that the extraction procedures selected for this work have been reported to minimize aggregation of copper-binding proteins (Ryden and Deutsch, 1978). The distribution of the two proteins was also organ-specific. Copper occurred on "copper-binding protein" in gills and muscle and on metallothionein-like protein in kidneys and viscera. The large quantities of metals on the latter were consistent with the role of kidneys and viscera (which included digestive gland) as storage or excretory organs for trace metals (Coombs and George, 1978; Schulz-Baldez, 1978; Lowe and Moore, 1979). Further biochemical characterization will be required to resolve the apparent differences in the properties of the two proteins.

Regardless of the identities of the copper-binding proteins in *Protothaca staminea* as possibly copper-chelatin or metallothionein-like, it appears evident that such macromolecules must play an important role in the regulation of intracellular copper. Their presence in control clams suggests a function in the routine metabolism of copper. Exposure of clams to copper resulted in increased copper levels on those proteins. Binding of copper to the proteins may have served a protective function by removing that fraction of copper from the general intracellular environment. The appearance of copper in the high molecular weight protein pool in exposed clams is consistent with the notion of "spillover" of toxic metals from low molecular weight "detoxification" proteins to high molecular weight substances during exposure of organisms to toxic concentrations of metals (Winge, Krasno, and Colucci, 1973; Brown, Bawden, Chatel and Parsons, 1977; Brown and Parsons, 1978; Engel and Fowler, 1979).

The limpet *Patella vulgata* (Howard and Nickless, 1977a) and mussel *Mytilus edulis* (Viarengo, Pertica, Mancinelli, Palmero and Drunesu, 1980) have been reported to possess a metallothionein-like copper binding protein. In other molluscan species such as the oyster *Ostrea edulis* (Coombs, 1974; Howard and Nickless, 1977b) and periwinkle *Littorina littorea* (Howard and Nickless, 1978), copper in soluble fractions has been shown to be associated mainly with small molecules such as amino acids and the betaine homarine, not with metal-binding proteins. There appears to be great diversity among related species in the mechanisms for intracellular storage of excess copper, although the use of small organic chelators remains as a common strategy. Further investigations into the mechanisms for the uptake and storage of trace metals will aid in the understanding of both the metabolism and toxicity of those substances.

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Summary

1. Clams of the species *Protothaca staminea* were exposed to a range of copper concentrations of control (= 0.35), 7, 18, 39 and 82 μg/l for 30 days.

2. Mortalities were monitored during the exposure. The results indicated that copper concentrations of 39 and 82 μg/l were extremely stressful with survivals of only 14 and 3%, respectively, after 30 days. At 7 and 18 μg/l, survival was slightly reduced when compared to controls.

3. Analyses for tissue copper concentrations showed that the gill was the primary organ for the concentration of copper and exhibited a linear relationship between its copper content and the exposure concentration.

4. Effects of copper on the gill were demonstrated by a disruption of sodium and potassium regulation at 39 μg/l and increases in acid phosphatase activity at lower exposure concentrations. The former was associated with high mortalities, while the latter was considered to be a sublethal cytotoxic response.

5. Two low-molecular-weight, copper-binding proteins could be distinguished by differences in their eluting properties. In gill and muscle, copper was associated with a protein of approximately 14,000 daltons, while zinc was associated with a protein of approximately 10,500 daltons. In viscera and kidney, copper and zinc co-eluted on the smaller of the two proteins and, in addition, considerable amounts of cadmium were detected on the kidney low molecular weight protein. The estimated molecular weight of the smaller protein and its ability to bind copper, zinc, and cadmium are similar to properties described for metallothionein.

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EFFECTS OF JUVENILE HORMONE ON ECDYSONE-DEPENDENT DEVELOPMENT IN THE TOBACCO HORNWORM, *MANDUCA SEXTA*

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Regulation of ecdysone production by insect prothoracic glands (PG) is generally considered to be the province of the brain and its prothoracicotropic hormone (PTTH). The insect life cycle is often viewed as a series of hormonally silent periods punctuated by bursts of PTTH secretion which drive production of ecdysone by the PG, thereby triggering molting and metamorphosis. Juvenile hormone (JH) is generally considered to have primarily morphogenetic effects which are most often manifest as a retardation of metamorphosis with maintenance of the *status quo*.

In addition to this role, JH is known to exert effects on the rate of ecdysone-dependent events in some systems. JH has long been known to be capable of provoking the development of diapausing saturniid pupae (Williams, 1959; Gilbert and Schneiderman, 1959). Hiruma, Shimada, and Yagi (1978) described a similar tropic effect of JH on the development of neck-ligated mature larvae and of brainless pupae in the noctuid *Mamestra brassicae*. In all these cases the authors concluded that JH exerted its stimulatory effect on the PG. By contrast, Nijhout and Williams (1974) demonstrated that JH delayed the onset of wandering in intact mature fifth-instar larvae of *Manduca sexta*. This result was interpreted as an inhibitory effect of JH on the release of PTTH by the brain, thus indirectly blocking ecdysone secretion by the PG.

In the present study we sought to clarify these seemingly contradictory effects of JH by further examination of the actions and interactions of the brain, the corpora allata (CA), and the PG in the postembryonic development of *Manduca*. The findings presented here direct attention to a previously unsuspected shifting role for JH in the control of ecdysone's secretion, metabolism, or action.

MATERIALS AND METHODS

Larvae were reared at 25° C on an artificial diet, as described by Bell and Joachim (1976) and Truman (1972), under either short-day (SD, 12 hr light: 12 hr dark) or long-day (LD, 17 hr light: 7 hr dark) photoperiods. Time of day was arbitarily referenced to lights-off at midnight (24:00 = 00:00). Larvae of the following types were segregated early in the photophase of each day: freshly ecdysed fourth-instar larvae and pharate fourths showing slipped head capsules, freshly ecdysed fifths, and fifths showing freshly exposed dorsal vessels. The timing of events in the life cycle was as described by Truman (1972) and Truman and Riddiford (1974) except that the first 24 hr of each stage was termed Day 1 rather than Day 0.

Neck ligatures were placed between the head and prothorax of larvae and between the head and prothoracic spiracles of pupae within 6 hr after pupal ecdysis. Isolated abdomens of larvae were obtained by placing ligatures across the first

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abdominal segment or between the metathorax and first abdominal segment. In all cases the portion anterior to the ligature was excised. Ligatures were of cotton-covered polyester (J. & P. Coats). Individuals were anesthetized with CO_2 prior to ligation and the surgical procedures.

Operations other than ligation were carried out on individuals immersed in insect Ringer's solution (Ephrussi and Beadle, 1936). Manipulations of the larval brain were performed through a small flap cut to one side of the midline just above the juncture of the cuticular sutures of the head capsule. The brain was easily located and gently drawn toward the incision by the entering bundles of trachea and nerves. These bundles were severed with micro-scissors and the brain withdrawn. Pupal brains were removed through a horizontal incision on the vertex of the head. All surgical incisions were sealed by apposition of the cut edges, thorough blotting of the region surrounding the incision, and application of a small drop of melted Tackiwax (Cenco). Less than 5% of individuals failed to survive the operations, and these typically died within 1–3 days. They have been excluded from the data presented.

The JHA "Hydroprene" (Zoecon, ZR-512) was used in the experiments described; all major findings were confirmed and duplicated with C18-JH (JH-1) and the JHA "epoxygeranyl sesamol" (both from Eco-Control). Hormones were dissolved in reagent grade acetone and applied to the dorsal thorax with a 100 μl Hamilton syringe on a repeating dispenser.

Results

Effects of JH on the metamorphsis of neck-ligated larvae

Once *Manduca* larvae in the fourth or fifth instars have attained a critical weight peculiar to each instar, they are able to develop following neck ligation (personal observations). Whenever such development takes place, it consists not of a larval molt such as one would anticipate of the fourth instar, but of exposure of the dorsal vessel—a distinctive prodrome of pupation. Indeed, those individuals surviving for a week or more after heart exposure usually proceed to form headless pharate pupae which in some cases undergo successful ecdysis.

A homogeneous group of Day-3, second-gate, fourth-instar larvae were neck-ligated at 20:00–21:00 and treated with JHA at doses ranging from 0.0001–1 μg. As shown by the hatched line in Figure 1, individuals receiving subthreshold doses exposed their dorsal vessels after an average of 4 days. Above the threshold dose of 0.05 μg, the delay was proportional to the logarithm of the dose. In additional experiments we found that neck-ligated fourths receiving 2.5 μg or more never exposed their dorsal vessels, nor did they show any other sign of development during their 1–2 weeks of survival.

The effect of JHA application was next examined in a group of second-gate LD fifth-instar larvae which were neck-ligated at 20:00–22:00 on Day 4. Here again (Fig. 1), application of JHA in doses above a critical level (in this case *ca.* 0.5 μg) delayed exposure of the dorsal vessel. The highest dose tested delayed heart exposure about 10 days beyond controls. In a further experiment not shown in Figure 1, daily application of 10 μg of JHA to similar neck-ligated larvae prevented any further development during their 3 to 4 weeks of survival.

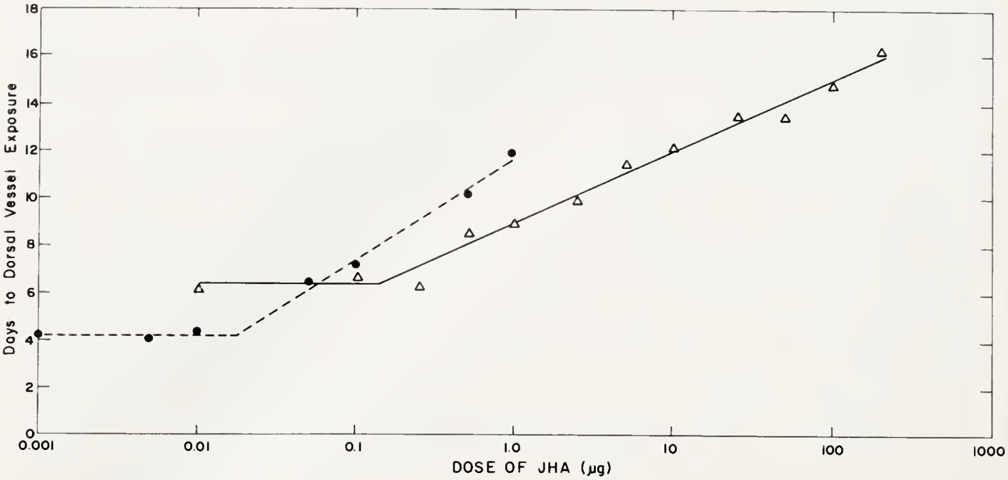

FIGURE 1. Effects of JHA application on the time to dorsal-vessel exposure of neck-ligated fourth- (closed circles) and fifth- (open triangles) instar larvae. Fourth-instar larvae weighed 0.75–1.0 g and were ligated between 20:00 and 21:00 AZT. Fifth-instar larvae weighed 7.0–8.5 g and were ligated between 20:00 and 22:00 AZT on Day 4 of the instar. Each point represents an average of the time to dorsal-vessel exposure of those 12–25 larvae which eventually exposed the dorsal vessel. At the higher concentrations some larvae in both instars died from starvation and desiccation prior to dorsal-vessel exposure. Standard deviations ranged from ±1 to ±2.5 days and tended to be larger at the higher doses.

Effects of JH on the development of brainless larvae

We have previously observed (Safranek and Williams, 1980) that when the brains of second-gate fourth-instar larvae were extirpated prior to 23:00 on Day 3, none of the brainless larvae showed any development during the remaining 1.5–3 weeks of survival. The same was true when the experiment was repeated on a similar group of 20 brainless fourths which received, in this case, daily applications of 20 μg JHA following brain removal. Yet when another group of brainless larvae were neck-ligated several days after brain removal, many of the individuals exposed the dorsal vessel within a few days. This could be prevented by application of a single dose of 20 μg JHA immediately after neck ligation.

Similar experiments were performed on mature fifth-instar larvae (second-gate Day-4 LD larvae weighing 7.0–8.5 g at 19:00–23:00) approximately 30 hr before they would have undergone dorsal vessel exposure and initiated wandering behavior. The brains were removed from 36 larvae and another 15 were sham-operated. Each individual was then placed in a plastic container without food. Eighteen of the brainless larvae received daily applications of 2 μl of acetone. These all initiated wandering after 4.1 ± 1.7 (standard deviation) days. The other 18 brainless larvae as well as the 15 sham-operated larvae received daily applications of 20 μg JHA. The sham-operated larvae all molted to sixth-instar larvae 5.4 ± 1.1 days following operation. By contrast, the JH-treated brainless larvae never undertook development of any kind although they survived for 21.0 ± 7.1 days. Additional experiments demonstrated that as little as a single dose of JHA administered to brainless fifths could provoke a substantial delay in metamorphosis. For example, topical application of 200 μg of JHA immediately after

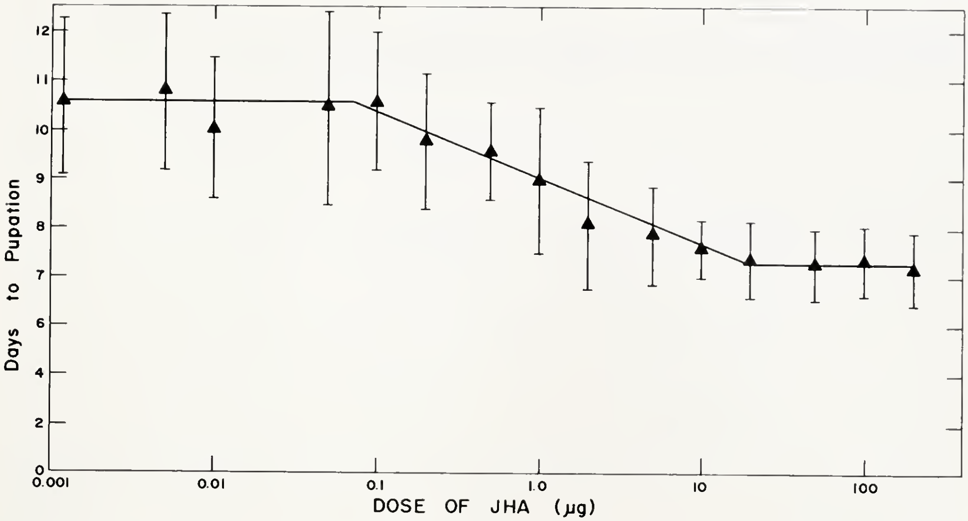

FIGURE 2. Effects of JHA application on the time to pupation of neck-ligated wandering larvae. Larvae were ligated between 07 : 00 and 12 : 00 AZT on the first day of wandering. All groups contained roughly equal numbers of first- and second-gate larvae. Points represent averages of the times to pupation of 15–20 larvae. Larvae which died prior to pupation were not included. This mortality, which ranged up to 30%, was highest at the lowest concentrations shown. Vertical bars equal ± standard deviation.

removal of the brain as late as 12 : 00 on Day 5 of the final instar often delayed the onset of dorsal vessel exposure by more than a week.

Effects of JH on neck-ligated and brainless wandering larvae

Up to this point we have considered the effects of neck ligation or brain removal, with or without JH treatment, on the initiation of metamorphosis as signaled by exposure of the dorsal vessel. We carried out the same experimental maneuvers on fifth-instar larvae which had already exposed their dorsal vessels and initiated the 2-day period of wandering. The effects were judged in each case by the time which elapsed between the treatment and the completion of pupal development, which was indicated by the formation of a tanned pupal cuticle.

In normal larvae reared at 25° C, an average of 5 days elapses between the onset of wandering and pupal ecdysis. This period was extended to an average of 10–11 days when neck ligation was carried out on the first day of wandering. Specific doses of JHA were applied immediately after neck ligation on the first day of wandering. The results, summarized in Figure 2, show that the delay occasioned by neck ligation was shortened in a dose-dependent manner. However, neither the highest single dose tested nor doses repeated daily were able to eliminate completely the delay resulting from neck ligation.

Substantially the same result was observed in parallel experiments carried out on larvae that had been neck-ligated on the second day of wandering (data not shown). Neck ligation after the second day of wandering caused little or no delay relative to unligated controls—a finding that suggests that the head's contribution to pupation is completed during the second day of wandering. Larval abdomens isolated by ligation on the first day of wandering survived for 1–3 weeks but showed

TABLE I

Effect of various procedures on the pupation of wandering-stage short-day larvae.

Procedure	Number of larvae	Average time to pupation (days ± s.d.)
No treatment	16	4.8 ± 0.2
JHA application	17	4.5 ± 0.2
Sham head operation	17	5.3 ± 0.2
Sham head operation and JHA application	17	4.9 ± 0.5
Brain removal	17	6.8 ± 0.7
Brain removal and JHA application	17	5.7 ± 0.3
Neck ligation	25*	10.3 ± 1.6
Neck ligation and JHA application	22	6.3 ± 0.5

* Four additional larvae died beyond 10 days after undergoing apolysis without apparent cuticle formation.

no further development. In the absence of the thorax, these abdomens could not be rescued even by large or repeated doses of JHA.

Removal of the brain from wandering larvae causes a delay of about 2 days in the onset of pupation (Safranek and Williams, 1980). Preliminary experiments suggested that JH might lessen this delay. Accordingly, a large number of synchronous Day–1 SD wandering larvae derived from a group of second-gate fifth instars were subjected to a variety of procedures to examine the effects of JHA application. All procedures were performed between 12:00 (Hour 0 for purposes of calculation) and 15:00. Individuals receiving JHA were treated with 200 μg of JHA immediately after ligation as well as on each of the subsequent 2 days. Because of the relatively small differences anticipated between groups, larvae were examined for pupation every 6 hr. The results of this experiment (Table I) show that the addition of JHA accelerated the pupation of all groups—even intact larvae. JHA reduced but did not eliminate the delay provoked by brain extirpation. Furthermore, the JHA treatment eliminated most of the very long delay induced by neck ligation.

Larvae treated with JHA after the onset of wandering ultimately formed normal pupal cuticle. This was anticipated on the basis of results of earlier experiments (Truman, Riddiford, and Safranek, 1974) which demonstrated the inability of JH to disturb normal pupal formation when administered after the initiation of wandering. So also, the morphology of pupae developing from brainless larvae was normal. Though some of the individuals failed to undergo ecdysis, this was apparently due to abnormalities in the head region secondary to the earlier operation. Many of the JHA-treated larvae formed healthy, intact headless pupae which ecdysed successfully and lived for several weeks. Even those which failed to ecdyse formed normal, well-tanned pupal cuticles. By contrast, headless larvae which did not receive JHA treatment eventually formed pupal cuticles which were recognizably pupal but otherwise thin, fragile, and often poorly tanned. Those few which successfully ecdysed had short wings and rarely survived more than a few days. Many of these abnormalities appeared to be the result, not so much of the absence of JH, as of the delay which resulted from the lack of JH. When untreated headless wanderers were kept in an environment whose atmosphere was saturated with water vapor, cuticular abnormalities seen previously were markedly reduced although the onset of pupation was still considerably delayed.

Effects of JH on the development of headless and brainless pupae

In a series of preliminary experiments, non-diapausing pupae were neck-ligated within 6 hr after ecdysis. Though these decapitated individuals failed to enter the expected extended diapause, their development was nevertheless always delayed by at least 2 weeks.

To examine the effect of JH, we decapitated several hundred potentially non-diapausing, recently pupated LD pupae and then applied a specific dose of JHA to each individual. The development of the treated populations was examined after two weeks. As shown in Figure 3, the JHA treatment accelerated development in a dose-dependent manner. All individuals initiating development within the first 2 weeks showed abnormal characteristics attributable to JH, as described previously by Riddiford and Ajami (1973).

Diapause-destined pupae derived from SD larvae were subjected to brain extirpation within 12 hr after pupation. As was shown by Safranek and Williams (1980), all such individuals typically require at least 8 weeks to initiate development. One week after the operation 25 brainless pupae and 25 1-week-old unoperated diapausing pupae were treated with 200 μg of JHA. When examined 2 weeks later, development had been initiated by 24 of the brainless pupae and 19 of the unoperated controls.

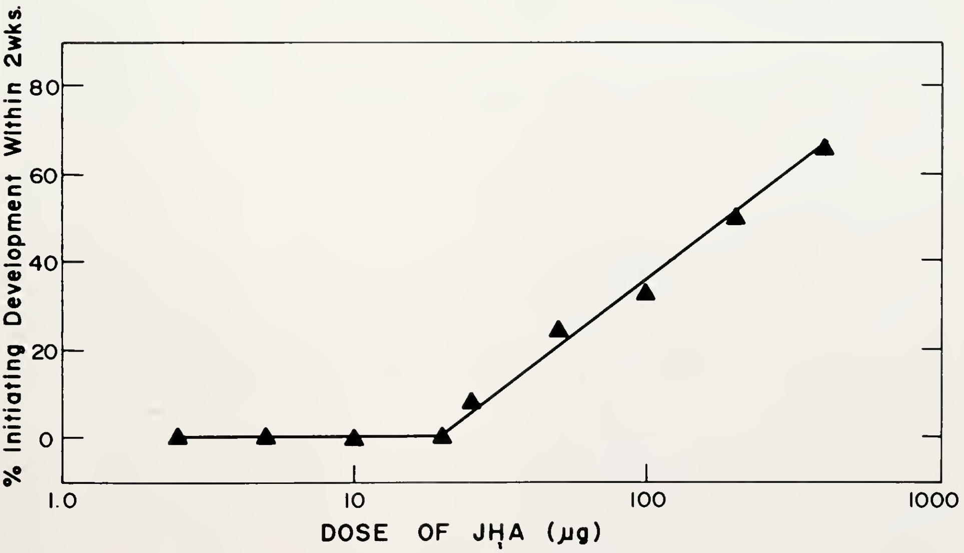

FIGURE 3. Effects of JHA application on initiation of development by neck-ligated pupae. Non-diapausing pupae were ligated within 6 hr after pupation. Initiation of development was recognized by apolysis of the wing epidermis. The time to completion of development from this point did not differ from that seen in unligated controls. Groups of 15–18 pupae were utilized at each dose tested. The data at two weeks are presented because some ligated controls initiated development during the third week. All pupae eventually developed after many weeks.

Discussion

JH accelerated the development of tobacco hornworms from the very onset of the wandering stage. This was true not only of intact wandering larvae and pupae but of brainless and neck-ligated individuals as well. The report by Hiruma, Shimada, and Yagi (1978) of a similar pattern of response by wandering larvae and pupae of the noctuid *Mamestra brassicae* suggests that the potential for JH stimulation of development may be widespread among mature larval and pupal Lepidoptera.

Mature larvae of the fourth or fifth instar initiate metamorphosis following appropriately timed neck ligation. The most novel finding in the current study is that prior to the onset of the wandering period, as signaled by exposure of the dorsal vessel, the metamorphic development of such neck-ligated larvae is actually delayed or inhibited by JH. Though this effect of JH is lost once the brain has provoked the initiation of a larval molt or of wandering, application of JH to larvae neck-ligated even a few hours before this time was able to delay or completely prevent any subsequent development.

Fifth-instar larvae whose brains were extirpated late in the instar underwent metamorphosis as did their neck-ligated counterparts. And as in the case of neck-ligated larvae, this development could be delayed or prevented by JH application. In contrast to this metamorphic development of brainless fifth-instar larvae, brainless fourths underwent a complete developmental arrest. When the brains were extirpated from fourth-instar larvae which would have exposed the dorsal vessel had they been neck-ligated, the larvae never underwent any type of development. This outcome was not altered by treatment with JH. The developmental inactivity of these larvae therefore resembled that of the neck-ligated larvae treated with higher doses of JH. Indeed, when such brainless larvae were neck-ligated even several days after brain removal, they proceeded to initiate metamorphosis, as signaled by exposure of the dorsal vessel. As with other neck-ligated larvae, this developmental response could be prevented by the application of JH. These observations are consistent with a continued secretion of JH by the CA of brainless fourth-instar larvae. By contrast, the initiation of dorsal–vessel exposure by brainless fifths indicates that the JH in these larvae routinely declines to levels permitting the onset of metamorphosis.

The ability of JH to delay the onset of wandering in intact mature fifth-instar larvae has been previously noted (Nijhout and Williams, 1974). This result was interpreted as an inhibitory effect of JH on the brain's release of PTTH. However, the present study has demonstrated that the pronounced ability of JH first to delay and subsequently to accelerate development persists in brainless individuals. Consequently the brain and its PTTH cannot be the sole targets for these effects of JH, if in fact they are targets at all.

Although isolated abdomens do not respond to JH application, injections of α- or β-ecdysone can cause them to undergo larval molting, dorsal–vessel exposure, pupation, and adult development (Fain and Riddiford, 1976; Nijhout, 1976; personal observations). Ecdysone must therefore be the ultimate vehicle for the various responses of neck-ligated larvae and pupae to JH application. JH might intervene in one or more of the following processes: 1) α-ecdysone synthesis and secretion by the PG; 2) conversion of α-ecdysone to β-ecdysone; 3) ecdysone inactivation and excretion; 4) response at the cellular level to ambient ecdysone. These several possibilities will be examined in subsequent communications in this series.

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SUMMARY

1. Juvenile hormone delayed or prevented the onset of metamorphosis by neck-ligated fourth-instar tobacco hornworm larvae and by fifth instars neck-ligated prior to the cessation of feeding.

2. After the onset of the wandering period JH had precisely the opposite effects in that it accelerated the onset of metamorphosis. This was the case both for wandering larvae and for pupae, irrespective of whether they were intact or subjected to brain removal or neck ligation.

3. These findings point to a previously unsuspected shifting role of JH in the control of metamorphosis.

4. Fourth-instar larvae underwent no further development after brain removal unless they were also effectively allatectomized by neck ligation. Evidently the secretion and inhibitory action of JH persists in brainless fourth-instar larvae.

5. Brainless fifths, by contrast, were often able to initiate metamorphosis despite the continued presence of their CA. Development could be prevented by daily application of JH. The disappearance of effective levels of JH, which normally occurs in the late fifth instar as a necessary prelude to metamorphosis, can evidently take place in the absence of the brain. This implies that the brain is not prerequisite for the decline in JH.

6. Since the ability of JH first to delay and subsequently to accelerate the development of intact individuals also persists after brain removal, the brain and its PTTH cannot be the sole targets for these effects of JH, if in fact they are targets at all.

7. The shifting effects of JH on development appear to be ecdysone-dependent. Four possible sites of JH action are identified.

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GAMETOGENESIS AND EARLY DEVELOPMENT OF THE TEMPERATE CORAL *ASTRANGIA DANAE* (ANTHOZOA: SCLERACTINIA)

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The growing interest in coral reefs and coral biology has not led to many studies of reproduction. Recent reviews of coelenterate reproduction and development (Campbell, 1974; Connell, 1973; Mergner, 1971; Miller and Wyttenbach, 1974; and Uchida and Yamada, 1968) have dealt mainly with Scyphozoa and Hydrozoa, and other anthozoan groups such as the anemones. While it seems likely that patterns of reproduction in corals may be similar to those in anemones, we lack detailed studies of the corals themselves. Our present knowledge is largely based on the studies during the Great Barrier Reef Expedition by Marshall and Stephenson (1933), who found sporadic occurrences of gonadal tissues and reproductive products in most of the species examined. For some species they were only able to locate individuals of one sex. Several species of *Pocillopora* were shown to brood their embryos up to the planula stage, but early stages of embryogenesis were not observed. Subsequent studies on coral reproduction have been limited to observing the release of planulae (Stimson, 1976, 1978; Harrigan, 1972) or the development, settlement and metamorphosis of planulae after release by the parent colony (Abe, 1937; Atoda, 1947, 1951; Lewis, 1974). Recent studies by Rinkevich and Loya (1979, a, b) on reproduction of the Red Sea coral *Stylophora pistillata* include some histological information on gonadogenesis but emphasize the ecological aspects of reproduction in this species. They do not report observing the transition from ovum to planula. No systematic studies have described gametogenesis, spawning, fertilization and early embryogenesis in any coral species.

This report presents the results of an 18-month study of the reproductive activities of the temperate scleractinian coral *Astrangia danae* living near the northern limit of its geographic distribution. Along the southern end of Narragansett Bay, Rhode Island, *A. danae* occurs as small encrusting colonies on rocky surfaces and shells (Agassiz and Fewkes, 1889). It is found in nature both with and without the endosymbiotic dinoflagellates known as zooxanthellae and is remarkable among corals in tolerating an annual temperature range of −2° C to 22° C.

MATERIALS AND METHODS

Collection

Colonies of *A. danae* were collected by SCUBA divers using chisels and knives to remove the corals from their rocky substrate. They were placed in plastic

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Table I

Criteria for differentiating stages of gonadal development in Astrangia danae, *as seen in histological sections of the mesenteries.*

Stage	Male	Female
O	No spermaries in mesentery; completely dispersed spermatocytes	Degenerate ova
I	Small clusters of interstitial cells in mesoglea of mesentery	Enlarged interstitial cells with large nuclei in mesoglea of mesentery
II	Spermary boundaries distinct but spermatocytes with small nuclei	Accumulation of small amount of cytoplasm around nuclei
III	Spermatocytes larger with large nuclei	Increasing amount of cytoplasm around nuclei but no vitelline membrane
IV	Spermatocytes undergoing meiosis and nuclear condensation but tails not evident	Oocytes full size with vitelline membrane; chromatin dispersed
V	Spermatozoa with tails; ready to spawn	As in stage IV but chromatin condensed; seldom seen
S	Spawned spermary	——

bags containing sea water and returned to running seawater aquaria where they were maintained at close to the temperature of the bay. Specimens used for histological examination were fixed within 36 hr of their return to the laboratory.

Histological preparation

Several different fixatives were tried, including Bouin's and Fleming's (Gray 1954) and a modified Helly's (Yevich and Barszcz, 1977). The best results were obtained by overnight fixation with Helly's fixative; it was more effective in retaining all of the many types of intracellular granules found in the gastrodermal tissues. Primary fixation with Helly's for 2 hr followed by post-fixation with a mixture (1:1) of Helly's and 2% OsO_4 was also used during the latter part of the study and was superior for fixing lipid reserves and oocyte yolk deposits.

After fixation, the corals were decalcified for 24 hr in Decal (Harleco Co.) and rinsed for 24 hr in running tap water. Dehydration, clearing and paraffin infiltration were done with a Technicon Tissue processor using Technicon reagents S-29 and UC-670 for dehydration and clearing and Paraplast for infiltration and embedding. Initial problems with tissue cracking were solved by reducing the three infiltration steps from 30 min to 15 min each.

Tissues were step-serially sectioned, 4–7-μm thick, and stained with Harris' hematoxylin and eosin (H & E) or Heidenhain's aniline-blue (Luna, 1968).

Quantification of gonad stages

A system of six spermary stages and five oocyte stages (Table I) was used to quantify the histological observations of gonad developmental stages. Five mesenteries were selected from each colony sectioned and the number of each stage of spermary or oocyte was counted. These data will be included in the doctoral dissertation of A.S.F. (University of Rhode Island, expected date 1980). The percent occurrence of a given stage was then calculated for each sex for each sampling date (Fig. 1). An example of typical data and of the method used in calculating the results is presented in Table II.

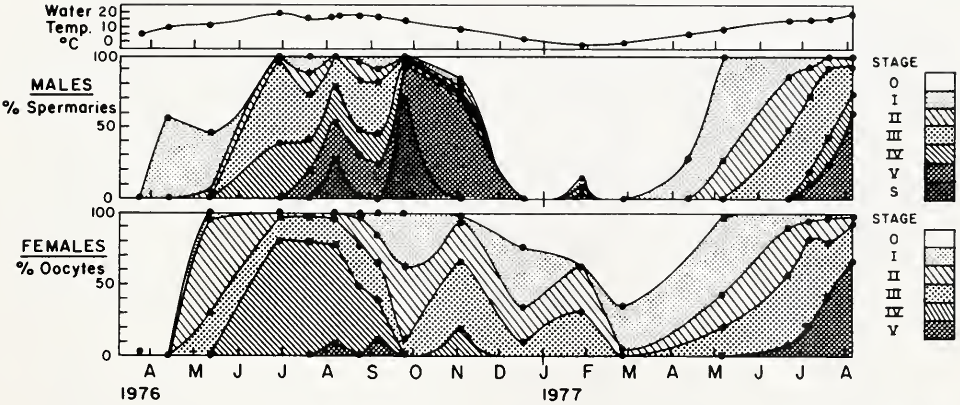


FIGURE 1. Temporal changes in the development of the gonads of *Astrangia danae* in Narragansett Bay. The stages (defined in Table I) were calculated from counts made on histological sections of the mesenteries. Temperatures reported were taken at the collection sites.

Spawning and fertilization

Live coral colonies were sexed by examining reproductive products in freshly collected bits of gonadal tissue. Fine-tipped forceps were inserted through the mouths of individual polyps and small pieces of mesenterial tissue removed for examination under a compound microscope.

Sexed colonies were placed in individual finger bowls filled with filtered sea water. Spawning was induced by placing small pieces of male gonadal tissue inside female polyps and female tissue inside male polyps. Fertilization of the eggs was accomplished by pipetting the freshly spawned eggs into finger bowls and adding water from the bowls of spawned males. Water in the bowls with developing coral larvae was changed at least twice a day during the first 48 hr and once a day thereafter. Samples of the sperm, unfertilized and fertilized eggs, and various stages of embryonic development were observed and photographed with a Zeiss photomicroscope.

Additional samples of the various embryonic stages were fixed in sea water-glutaraldehyde (3%), post-fixed in OsO_4 (1% in sea water) and embedded in Spurr's medium (Polysciences, Inc., Warrington, Pa.). Silver sections were stained with 2% uranyl acetate and Reynold's lead citrate, and observed with a Zeiss electron microscope.

RESULTS

General

As in other anthozoans, the gonads of *A. danae* are located in the mesenteries, between the mesenterial filaments and the muscle bands (Fig. 2). Each polyp had up to 24 mesenteries, and all were capable of bearing gonads.

Colonies of different sizes were used in this study. The smallest ones collected, of from 6 to 9 polyps (estimated age of 1 to 3 years, based on growth rates in the laboratory), were already capable of sexual reproduction.

TABLE II

Gonad-stage data for colonies collected on June 20, 1977: an example of typical data and of the method used to calculate the average percent occurrence of each stage seen in Figure 1.

Colony No.	Sex	Mesentery Number																			
		1				2				3				4				5			
		Stage				Stage				Stage				Stage				Stage			
		I	II	III	IV	I	II	III	IV	I	II	III	IV	I	II	III	IV	I	II	III	IV
45	M	4		2		3				9	3			4	3			1	6		
46	M	5	5	12		2	5	4			14	20		3	8	2		6	24		
47	F		17			11	18			12	17				4			4	7	6	
48	M		1	1				3													
49	M		2	1			4	4		1	1	3			3	1			2		
50	F		5	21	4			8			18	20			3	22	5		6	29	
51	M	3	5	4			6	1			2	14		1	4	9		2	1	3	
52	F	1	6	11			3	9	4			28	8	1	6	9	3	2	4	9	6
53	M	2	4	29			4	12	3		11	10		1	2	11		3	10	23	
54	F	2	5	6	2	3	5	7			2	7			7	7	1	1	5	11	3
55	F	3	6	7		2	2	3	2		2	5	1	3	2	13				7	
56	F		7	1		3	5	2	1		1		4	1	9	13		7	3	7	
57	F		6	7																	

	Total staged	Average percent each stage on June 20, 1977						
		0	I	II	III	IV	V	S
Oocytes	556	0	10	33	49	8	0	—
Spermaries	352	0	14	37	48	1	0	0

All polyps belonging to a given colony were of the same sex; only one hermaphroditic polyp was found out of about 5000 polyps observed. Of the 153 colonies sectioned for this study, 3 had no recognizable gonadal material in the sections examined. Of the remaining colonies, 77 were male and 73 female, indicating a 1 : 1 sex ratio for this species.

An egg production estimate of 6000 eggs per polyp was obtained by counting the number of eggs present in vertical and cross sections of several polyps from a single colony. The polyps of this particular colony seemed to have a higher than usual egg density; the average egg density for this species is probably closer to two-thirds to one-half of this value.

Seasonal trends

Many stages of gonad development were simultaneously present in mesenteries of the same polyp, and such differences could also be seen between polyps in one colony and between colonies collected on the same date (Table II). Nevertheless, when the average percent occurrence of each stage is plotted (Fig. 1) clear seasonal trends are apparent.

Gametogenesis started in the early spring (March–April) and proceeded through June and July. The spawning season was from early August through September, when water temperatures were at their peak. In September 1976 a second cycle of gametogenesis started, but no evidence of a second spawning episode was encountered. Finally, the remaining unspawned gametes gradually disappeared over the winter months.

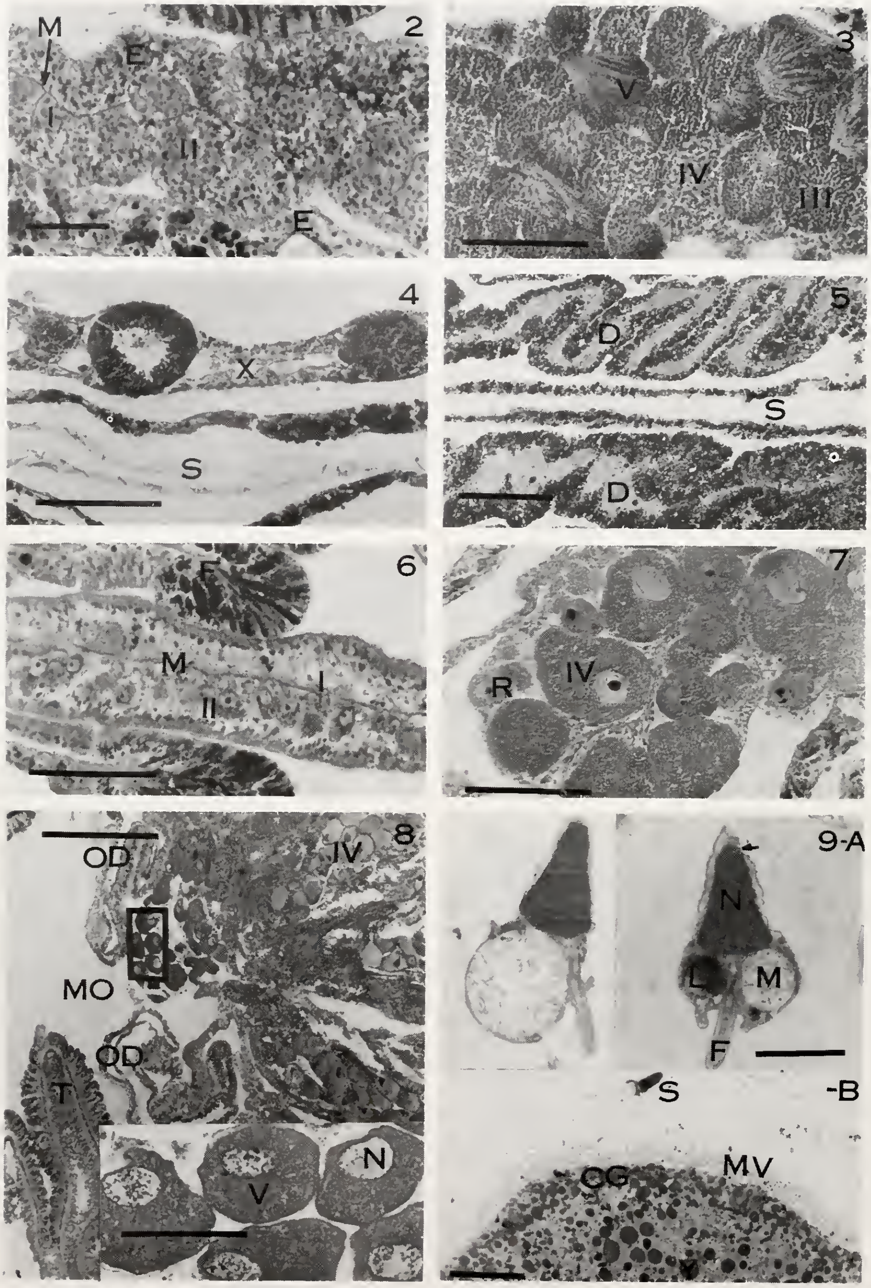
Separate descriptions of the gametogenic cycles of male and female colonies follow.

Spermatogenesis

It is difficult at this level of observation to distinguish between primordial germ cells and spermatogonia, and the transition of the latter into spermatocytes (Giese and Pearse, 1974). Therefore, the term spermatocyte was used to identify all stages of differentiation before the appearance of the spermatozoan tails.

Spermary formation began in the early spring with the appearance of interstitial cells (Hyman, 1940; Campbell, 1974) in the mesoglea. Here the interstitial cells formed small membrane-bound aggregates (stage I) (Fig. 2). The number and size of the cells in these early spermaries gradually increased, but the spermatocytes still retained an appearance similar to that of the initial interstitial cells (stage II). The spermatocytes then changed in staining characteristics, developing a greater affinity for hematoxylin, an indication of increased nucleic acid content (stage III). The spermatocytes became much more numerous and smaller, with condensed nuclei; however, spermatozoan tails could not be distinguished in the preparations (stage IV) (Fig. 3). Finally long tails became evident, and the spermatozoa appeared ready to be spawned (stage V) (Fig. 3).

Spawning resulted in the appearance of empty pockets in the male gonads where the spermaries had once been (stage 5) (Fig. 4). The major period of spawning during August and early September 1976 was followed by the reappearance of large numbers of early stage spermaries (I and II) in the mesenteries. By November late stage spermaries (III–V) were seen in many polyps, but there was no evidence that the spermatozoa were being released. Instead, by December



FIGURES 2–8. Photomicrographs of histological preparations of tissues of *A. danae* illustrating the gametogenic sequences described in text. Tissues were fixed in Helly's fixative and stained with Heidenhain's aniline-blue. Abbreviations used: D—dispersed spermatocytes; E—endoderm; F—mesenterial filament; I—stage I; II—stage II; III—stage III; IV—stage IV; M—mesoglea; MO—mouth; N-nucleus OD-oral disk; R—partially resorbed oocyte; S—septal space; T-tentacle; V-stage V; X—spawned spermary.

FIGURE 2. Cross-section of gonad-bearing portion of a mesentery with spermaries in developmental stages I and II. Scale bar equals 50 μm.

or January the gonads typically looked like the one shown in Figure 5. The membranes surrounding the spermaries and the tails of the spermatozoa were no longer visible. Lesser numbers of spermatocytes were observed in later collections, so it appeared that these cells were being absorbed.

Oogenesis

The early detection of gametogenesis in the mesenteries of female colonies was difficult because it involved the recognition of single interstitial cells, rather than the aggregates of cells seen in male gonads. This may account for the apparently later initiation of gametogenesis by female polyps then by male polyps (Fig. 1).

Stage I oogonia were found along the mesogleal lamella, where they could be distinguished by their large nuclei and nucleoli (Fig. 6). The smallest oogonia seen were about 5 μm in diameter. Very little cytoplasmic material could be seen around the nuclei at this stage. Vitellogenesis started after the oogonia entered the mesoglea (stage II). As the oocytes continued to grow, both the cytoplasm and the nuclei increased in size (stage III). Oocytes were assigned to the stage III growth phase until they reached nearly their full size. The increase in size is dramatic: there is a 600-fold increase in volume. The source material for this increase is not known, but it appears that some smaller oocytes may have been resorbed as the larger oocytes grew (Fig. 7). The formation of the vitelline membrane, which consists of a layer of cortical vesicles just beneath the cell membrane, marked the attainment of stage IV (Fig. 7). The vesicles made the peripheries of the cells stain dark blue with both the H and E and the Heidenhain's stains. The now mature eggs apparently remained in a stage IV condition until spawned sometime in August and early September.

In a small fraction of observations, a situation described as stage V was seen. The nuclear material appeared condensed rather than dispersed (Fig. 8). This stage may represent part of a final meiotic reduction; however, the release of polar bodies was not observed. The low frequency with which this stage was encountered suggests that it lasted only a short time before the eggs were spawned. After spawning the remaining mesenterial tissues were very broken up and disorganized.

FIGURE 3. Oblique section through a mature male gonad with spermaries at various stages of development up to stage V. Scale bar equals 100 μm.

FIGURE 4. Longitudinal section of a male gonad containing partially and completely spawned spermaries (stage S). Scale bar equals 100 μm.

FIGURE 5. Longitudinal section of several male mesenteries showing absence of spermary membranes and dispersed, tail-less spermatocytes. Scale bar equals 200 μm.

FIGURE 6. Early oogenesis seen in a longitudinal section of a mesentery. Stage I and II oocytes are strung along the mesogleal lamella. Scale bar equals 100 μm.

FIGURE 7. Section through ovary showing mature oocytes with well developed vitelline membrane (stage IV). Notice that some of the smaller oocytes seem to be partially resorbed. Scale bar equals 100 μm.

FIGURE 8. Longitudinal section of a female polyp fixed while spawning. Compare the nuclear material of the spawned ova (insert) with that of the oocytes in Figure 7. Upper scale bar equals 500 μm; insert scale bar equals 100 μm.

FIGURE 9. Electron-micrographs of *A. danae* egg and spermatozoa. A. Longitudinal sections through spermatozoa show single mitochondrion and large lipid body. Notice less dense material anterior to the tip of the nucleus (arrow). Scale bar equals 1 μm. B. Cross section of outer portion of an unfertilized spawned egg. A layer of sub-cortical vesicles lies beneath the microvillous oolema. Yolk granules and mitochondria are abundant within the cell. Scale bar equals 5 μm. Abbreviations: CG—cortical granules; F—flagella; L—lipid body; M—mitochondrion; MV—microvilli; N—nucleus; S—spermatozoan; Y—yolk granule.

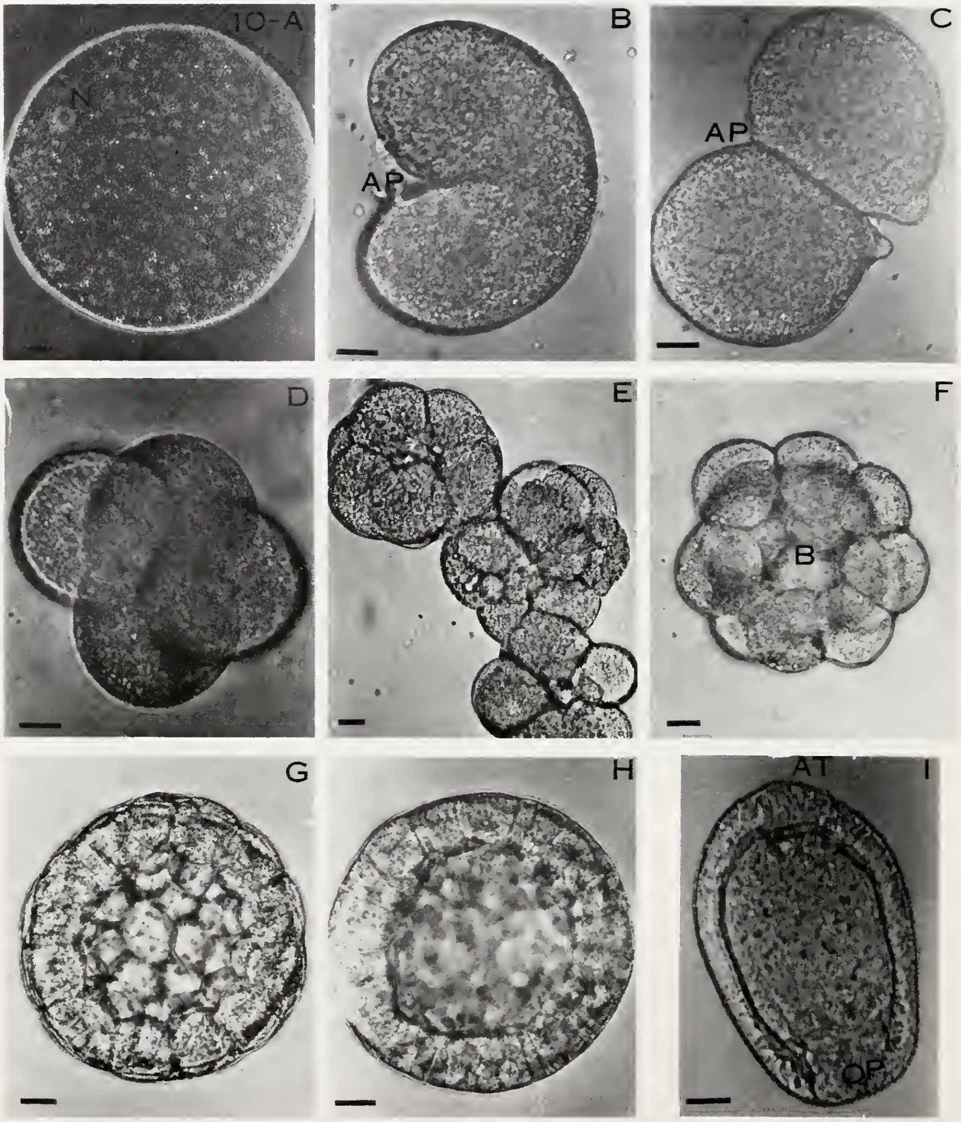


FIGURE 10. Photomicrographs of various stages in the embryonic development of *Astrangia danae*. A. Unfertilized egg wth eccentric nucleus. B. First cleavage proceeds from animal to vegetal pole. C. First division complete; notice small protuberances being pinched off at vegetal pole. D. Psuedospiral arrangement of blastomeres after second division. E. Irregularly shaped embryos resulting from nonsynchronous third and fourth divisions. F. Embryo of around 20 blastomeres, with large blastocoele. G. Sixty-four cell stage; blastomeres still rounded. H. Blastula stage; blastomeres compressed. I. Planula stage, showing apical tuft of cilia and oral pore. Abbreviations: AP—animal pole; AT—apical ciliary tuft; B—blastocoele; N—nucleus; OP—oral pore. Scale bars are 10 μm.

Few eggs remained in the gonads. By mid-September, however, stage I and II oocytes began to appear in the mesenteries again. Although some of these oocytes developed as far as stage IV, they were never as numerous and they never attained the size of the mid-summer oocytes. As with the male colonies, there was no evidence of a second spawning cycle. By December signs of oocyte absorption began to appear, such as the disappearance of yolk material. The process of absorption continued throughout the winter months until the unspawned oocytes were almost completely absorbed.

Spawning

Spawning was first observed in the laboratory when several colonies were inadvertantly subjected to heat and possibly low-oxygen stress. It was again accidently induced during the course of another experiment, possibly by low oxygen or a combination of unknown stress factors. Spawning was later experimentally induced, as previously described, by stimulating polyps of one sex with bits of gonadal tissue of the other sex. In all cases, a certain behavior pattern was noticed. The polyps first expanded to an extreme degree, but the tentacles remained short and stubby, very unlike the appearance of a polyp expanded in a feeding posture. The oral disk was very prominent, with the mouth tightly closed and puckered at the center. At the moment of spawning the polyp would suddenly shorten its column and a narrow stream of eggs or sperm would shoot out of its mouth. Within a few minutes the polyp would be fully expanded again, and the contraction with gamete ejection quickly repeated. Individual polyps were seen to go through this cycle up to six or seven times.

In most cases one polyp of a particular colony went through a couple of ejection cycles before any of the other polyps joined in. Not all polyps of a spawning colony spawned, and some seemed to be spawning more eggs or sperm than others.

The mature spermatozoa were around 2–3 μm in length, not including the tail. They were characterized by a single large mitochondrion and a large, electron-dense lipid body located at the base of the conically elongated nucleus (Fig. 9A). No acrosome was seen but, as can be seen in Figure 9A, there was an accumulation of moderately dense material anterior to the tip of the nucleus, similar to that reported by Hinsch and Clark (1973) for *Aurelia.*

The mature ova were around 100 to 130 μm in diameter, with an asymmetrically located nucleus (Fig. 10A). The ooplasm was densely populated with yolk bodies and mitochondria. The surface of the cell was microvillous, with a subsurface layer of cortical granules (Fig. 9B).

Fertilization and development

Most of the spawned eggs quickly settled to the bottom of the culture dish, where they could be pipetted and concentrated before fertilization. Figure 10 presents a sequence of photomicrographs of progressive stages of development of *A. danae* embryos. No fertilization membrane could be distinguished by light microscopy. The first division took place within the first hour after fertilization (AF). The cleavage furrow started at the animal pole (near the asymmetrically located nucleus) and progressed inward towards the vegetal pole (Fig. 10B). An event frequently seen at this time was the pinching off of two small portions of cytoplasmic material at the vegetal end (Fig. 10C). We have no explanation of this phenomenon. The timing of subsequent divisions varied between batches of eggs obtained on different occasions, but they were generally 30 to 60 min apart. The second division was always synchronous between the two blastomeres. The cleavage plane was radial but the blastomeres rearranged themselves after the division into a pseudospiral (Mergner, 1971) formation (Fig. 10D). Subsequent divisions were not always synchronous, resulting in a variety of embryos of different cell numbers and shapes (Fig. 10E). A blastocoele formed early in the cleavage sequence (Fig. 10F); blastocoeles were observed in embryos with as few as 12 blastomeres. At the *ca.* 64-cell stage, the blastomeres were still fairly rounded, especially on their inner surface (Fig. 10G). By the *ca.* 128-cell stage the blastomeres were closely spaced and columnar in shape (Fig. 10H). Ciliation was first seen at this time. Until now the embryos had remained on the bottom of the culture dishes. As ciliation increased, and with the advent of elongation and gastrulation the embryos began to swim and congregate at the surface of the dishes. At this stage, 6 to 8 hr AF, they became strongly attracted to light.

It is not clear by what process gastrulation took place. However there are some indications from the photomicrographs of the living embryos that gastrulation took place by primary delamination or mixed delamination (Mergner, 1971). The confirmation of this suggestion will have to await electron microscopy of the fixed embryos. No evidence of invagination or archenteron formation was seen in any of the embryos observed.

Within 12 to 15 hr AF the elongated gastrulae developed oral pores and apical ciliary tufts (planula stage) (Fig. 10 I). The young planulae swam and spun on their longitudinal axes quite rapidly, but still remained near the surface of the water. Over the next 24 hours the mouth and pharynx formed and the larvae became more elongated. From 48 to 72 hr AF the planulae became demersal or swam in mid-water; they no longer congregated at the surface, nor were attracted to light. The first signs of mesenterial development were noticed in a few of the larvae.

The embryos gradually decreased in size as development progressed. By the time the larvae reached the planula stage they were about 75 μm long—only $\frac{3}{4}$ to $\frac{1}{2}$ of the diameter of the original egg. Some of the culture dishes had an abundance of small flagellate, diatom and bacterial contaminants; but the planulae were never observed feeding on this material. Planulae in both "clean" and "contaminated" culture dishes shrunk at about the same rate. Attempts at presenting them with a variety of substrates failed to induce settlement, and evenutally all of the larvae died.

Discussion

What little is known about coral reproduction indicates that patterns of gametogenesis and development are quite variable (Connell, 1973; Rinkevich and Loya, 1979a). Previous studies have concentrated on mostly hermaphroditic species that brood their embryos and release a small number of large planulae (over 1 mm long) which settle and metamorphose soon after being spawned (Boschma, 1929; Abe, 1937; Marshall and Stephenson, 1933; Harrigan, 1972; Lewis, 1974; Stimson, 1976; Rinkevich and Loya, 1979a, b). Occasionally observations were made that indicated some coral species did not conform to this pattern of reproduction, but the information was too incomplete for alternate patterns of reproduction to be affirmed. The present study shows that *Astrangia danae* follows a different reproductive pattern: it is a dioecious coral, with large numbers of small eggs (*ca.* 0.1 mm diam.) and with external development. It is not clear at this time how these two different reproductive patterns, and their variants, are distributed among the ranks of coral families and genera, although Rinkevich and Loya (1979a) suggest that it may be related to gonad location within the polyp and polyp size. They suggest that small-polyped species have gonads that bulge into the gastric cavity, produce small numbers of small eggs and brood their larvae, while large-polyped species have gonads within the mesenteries and produce large numbers of large eggs which are spawned and fertilized externally. The available information for *A. danae* and for several species of Caribbean reef corals suggests that there is no universal relationship between polyp size, egg size, and mode of development. *A. danae* which has moderately sized polyps (3–5 mm in diameter) differs from the model (if classified as a large-polyped species) by producing a large number of small eggs. The genus *Favia* presents a more complicated picture. *F. doryensis* from the Great Barrier Reef (Marshall and Stephenson, 1933) and *F. favus* from the Red Sea (Rinkevich and Loya, 1979a) are large-polyped species that have mesenterially located gonads with many large eggs. Although planulation has not been observed for either species, it is well documented for the Caribbean species *Favia fragum* (Duerden, 1902; Lewis, 1974) which also has mesenterially located gonads, but with only one to five large ova per gonad (Duerden, 1902). Therefore, related species with similar polyp and egg size differ in the number of eggs produced and in the manner of development. Duerden (1902) also observed planulation by the large polyped species *Manicina areolata* and *Isophyllia radiata.* It is not surprising that many reproductive patterns occur among the scleractinian corals, in light of the extreme diversity of reproductive patterns found among the closely related sea anemones (Spaulding, 1974; Campbell, 1974; Chia, 1976).

As far as we know, this is the first report on gametogenesis and embryonic development of a coral species. There have been, however, several studies of this kind on sea anemones (Campbell, 1974; Clark and Dewel, 1974; Spaulding, 1974; Jennison, 1979), with which the observations on *Astrangia* can be compared.

The gametogenetic patterns observed are, as expected, similar to those reported for other anthozoans (Campbell, 1974; Jennison, 1979). The interstitial cells which give rise to the germ cells are of endodermal origin and migrate into the mesenterial mesoglea before starting to differentiate. The spermaries in *Astrangia* remain within the mesoglea throughout their development; however, it appears that as the oocytes become very numerous and large, they are pushed out of the mesoglea into the endodermal portion of the mesentery. Trophonemata, umbilical-like connections between the gastrovascular cavity and growing oocytes that have been found in various groups of anemones (Dunn, 1975; Jennison, 1979), were not seen in *A. danae.*

The mature *Astrangia* eggs are similar in size and appearance to those of the sea anemone *Metridium* (Clark and Dewel, 1974). The sperm, however, do not resemble in nuclear shape or mitochondrial position those reported for other anthozoan species (Campbell, 1974; Clark and Dewel, 1974; Hinsch and Clark, 1973; Hinsch, 1974). The lipid body associated with the single mitochondrion is much larger in *Astrangia* than those reportedly associated with the mitochondria of *Bunodosoma* and *Metridium* (Clark and Dewel, 1974).

Astrangia development differs from patterns observed for sea anemones with similarly small eggs (Spaulding, 1974; Campbell, 1974) in that the first cleavage always results in a two-cell stage, and gastrulation does not appear to occur by invagination. Further elucidation of the similarities and differences between *Astrangia* and other forms will have to await studies using both light and electron microscopy.

The reproductive cycle of *A. danae* in Narragansett Bay seems to be controlled by temperature, although control by food availability cannot be ruled out. *Astrangia* colonies maintained in the laboratory at 20–22° C and fed on a regular schedule were able to spawn year round. It should prove interesting to study the reproductive cycles of populations of *A. danae* living along the Gulf of Mexico and the coasts of Florida where water temperatures are relatively warm all year.

Astrangia danae is known to occur naturally both with and without zooxanthellae. It was of interest to determine in the course of this study whether in *A. danae* the zooxanthellae were transferred to succeeding generations through the eggs or larvae. In no section were zooxanthellae found inside of eggs, although the algae were often very abundant in the gonadal tissues. The absence of zooxanthellae in the eggs was confirmed with the observation of freshly spawned eggs from symbiotic female colonies. Attempts to infect planulae with algae extracted from adult colonies were unsuccessful. However, the failure of any of these planulae to settle under our maintenance conditions leaves unanswered the question of if and when the planulae take up zooxanthellae.

We wish to thank Mr. Wes Pratt for collecting specimens of *Astrangia* during the winter months and for providing much of the temperature data. Mrs. Carolyn Barszcz helped improve the histological preparation of the coral tissues. Dr. Elijah Swift V made available the Zeiss photomicroscope used for photography of the embryological stages, and Dr. Scott Nixon the microscope used in staging the histological preparations. We thank an anonymous reviewer for referring us to Rinkevich and Loya's recent papers. This work was supported by the E.P.A. Environmental Research Laboratory, Narragansett, and by NSF Grant OCE 75-18848.

SUMMARY

1. The coral *Astrangia danae* is dioecious, with an early age of first reproduction.

2. In Narragansett Bay, this species exhibits an annual reproductive cycle with gametogenesis starting in March–April. Spawning occurs during August, and vestigial gametes are absorbed during the winter months.

3. Gametes originate from interstitial cells which differentiate in the mesenterial mesoglea.

4. Ova are 100–130-μm in diameter and sperm are 2–3-μm in length, excluding the tails. Egg production was estimated at up to 6000 eggs per polyp.

5. Fertilization and development are external; no fertilization membrane was seen even though a layer of cortical vesicles was present before fertilization.

6. Embryonic cell divisions are around 30 min apart and the larval planual stage is reached within 12–15 hr after fertilization. Larval settlement was not observed.

7. Ova from colonies containing zooxanthellae did not contain zooxanthellae when spawned.

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PATTERNS OF SEXUALITY, ASEXUAL REPRODUCTION AND RECRUITMENT IN SOME SUBTIDAL MARINE DEMOSPONGIAE

AVRIL L. AYLING[1]

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Although there have been few careful studies on forms of sexuality in sponges, it is clear that sponge reproduction follows a number of patterns. Sponge species may have separate sexes (Chen, 1976) or partially separate sexes with varying numbers of hermaphroditic individuals (Elvin, 1976; Fell, 1976a). Other species are hermaphroditic, producing sperm and oocytes simultaneously (Sarà, 1961; Simpson, 1968; Chen, 1976), sequentially in the form of protogyny and protandry (Sarà, 1961, 1974; Diaz, 1973), or alternating sexes (Gilbert and Simpson, 1976). Sequential changes in sex may occur during a single reproductive season (Sarà, 1961) or over consecutive years (Diaz, 1973). Other characteristics of sexual reproduction that must be considered are: 1.) whether the sponges are viviparous with embryogenesis occurring within the adult, or oviparous with oocytes or zygotes shed into the sea, and 2.) whether sexual products are released synchronously or asynchronously in individuals or the entire population (Reiswig, 1976). In addition, a number of forms of asexual reproduction are found in sponges; for example, gemmule formation, budding, and fragmentation of the adult.

In only a few studies have gamete populations been quantified (Elvin, 1976; Fell, 1976a) or reproductive output related to larval release and settlement (Fell, 1976a; Fell and Jacob, 1979). The survival patterns of sponge larvae remain largely unknown.

One reason for the lack of reliable studies on sponge reproductive patterns is the difficulty of monitoring natural populations. Most intertidal sponges are subject to considerable physical disturbance, which affects their patterns of settlement, growth and reproduction (Wells, Wells and Gray, 1964; Elvin, 1976). Long-term monitoring of these populations is often difficult because of fluctuating abundances of the sponges. In some species, small size makes regular cutting of tissue samples difficult (Fell, 1970, 1974). However, subtidal sponge populations are not so subject to physical disturbances, and, as many of the species

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are long-lived (Dayton *et al.*, 1974; A. L. Ayling, 1978a), such populations can be sampled regularly by divers.

A second reason for the paucity of reliable studies is lack of procedures by which the type of reproduction may be rigorously established. Rather than sampling the same individuals regularly, over long periods, most studies have involved only sampling of the general population. This procedure by itself inadequately describes not only gametogenic cycles but often the type of sexuality.

The object of this study was to establish the reproductive pattern of 10 species of Demospongiae from the shallow subtidal waters off northern New Zealand. In addition, the reproductive output of the species was related to recruitment rates in the natural habitat and on artificial surfaces. If the young sponge successfully settled and survived through the first month of its life it was considered in this study to have been recruited into the population. Two groups of sponges were selected for the study: thinly encrusting ceractinomorph species, and structured tetractinomorph species such as spheres, fingers and massive spreading forms. These latter sponges are referred to as discrete species because they are easily recognizable as individuals in the field, whereas the thinly encrusting species were frequently fragmented. The reproductive patterns found in these species are discussed in the light of the ecological consequences of the various products produced.

Materials and Methods

The sponges investigated were located in two subtidal sponge dominated communities near the Leigh Marine Laboratory on the east coast of northern New Zealand (36°16′S: 174°48′E). Tissue samples of the thinly encrusting species were collected from "beach canyon," a steep-walled canyon 12 m in depth on the exposed northeast side of an island. These sponges covered 46% of the walls of the canyon and were regularly grazed on by the abundant urchin *Evechinus chloroticus.* They were also subject to heavy surges during storms. Tissue samples of the discrete sponges were collected from the "sponge garden," a flat rock platform covered by a thin layer of sediment at 18 m depth. Here, sponges covered only 14% of the substratum but were numerous and subject to very little disturbance (A. L. Ayling 1978a).

The abundance of the sponge species is given in A. L. Ayling (1978b) but the following nomenclatural changes have been made: *Polymastia granulosa* = *Polymastia* sp., *Cicocalypta penicillus* = *Polymastia* sp. (yellow), *Hymedesmia lundbecki* = *Stylopus* sp. and *Tedania* sp. (pink) = *Stylopus* sp. (pink).

Permanent buoys were set up to serve as reference points for mapping the locations of six individuals of each species. Each sponge was marked by hammering a nail bearing a numbered Dymo tape tag into the rock bottom beside the sponge. Small tissue samples of all marked sponges were taken fortnightly from June, 1976, to November, 1978, to establish reproductive periods and forms of sexuality. Counts of the numbers of reproductive elements were taken from two individuals per species throughout the reproductive period by cutting five sections from each tissue block and then relating the number of reproductive elements in the section to the area and thickness of the embedded tissue. The sponges appeared to be unharmed by the sampling procedure.

Tissue samples from large species were taken from several locations within the body of the sponge to check that single samples were representative of activity throughout the sponge. When most or all of these regularly sampled sponges were reproducing, a larger random sample of 25 or more sponges was collected, with records of the size of each sponge, to investigate activity in the entire population. Tissue samples were collected in small partitioned plastic containers and preserved in Bouins fixative. Sections were cut at 6 μm and stained with Erlich's Haematoxylin (30 min) and Eosin.

During the reproductive period, large pieces of tissue were collected and placed in running sea-water aquaria for observation of the number of larvae released per day per unit of tissue and the time the larvae took to settle. Sponges producing buds were individually tagged and the number of buds released over time recorded. The settlement and recruitment of larvae and buds on settlement plates, on cleared bare rock areas and in the natural habitat were monitored monthly using the following techniques: In both study areas asbestos board settlement plates, 0.0625 m square and 5 mm thick, were bolted to the rock each month. Also in each area five 0.0625-m squares were cleared of all encrusting organisms with a chisel and a wire brush and monitored over 2 years. In the natural habitat, eight 0.0625-m-square areas were set up in each area and monitored for 2 years. Additional information on discrete sponge recruitment was obtained from ten 1-meter-square areas of natural habitat in the "sponge garden." A 10-meter-square area was marked out and random pairs of numbers used to locate each quadrat. Then the outline of all sponges in each quadrat was traced onto acetate sheets using Chinagraph pencils. These drawings were repeated 1 year later. Additional information on larval recruitment in "beach canyon" was collected from 46 0.0625-m squares of natural habitat photographed at 3-month intervals. Small knobs of epoxy cement were used to mark out the corners of the quadrats.

RESULTS

Reproductive patterns in thinly encrusting ceractinomorphs

Stylopus sp. This orange sponge was the most abundant species in "beach canyon," forming extensive encrusting patches up to a meter in diameter and 5 mm thick, with smaller fragmented pieces surrounding the central bodies. *Stylopus* sp. reproduced over the summer months from late December or early January to the end of April. In the first year of the study, only three of the six continuously sampled sponges reproduced. In the following year, however, all the sampled sponges were active. This increase in the number of sponges reproducing was also reflected in the larger population samples, where at least 92% ($N = 70$) of sponges were active in 1978, while in the preceding year only 53% ($N = 50$) had been active.

Stylopus sp. is a simultaneous hermaphrodite which incubates its larvae. Early in the reproductive period spermatocytes appeared in numbers of up to 100 per mm^3. Then fertilized embryos appeared in numbers of up to 0.2 per mm^3 along the base of the sponge (Fig. 1A). Bright orange larvae emerged from single sponge patches over a period of approximately 2 months and were released at a rate of 1–4 per cm^3 of tissue per day. The larvae were spherical, 392 by 407 μm in size and covered with cilia except at the posterior pole, which was bare.

Stylopus sp. (pink). This thinly encrusting pink sponge was moderately abundant in "beach canyon," where some patches covered areas of over a meter square. *Stylopus* sp. (pink) is a simultaneous hermaphrodite similar in its re-

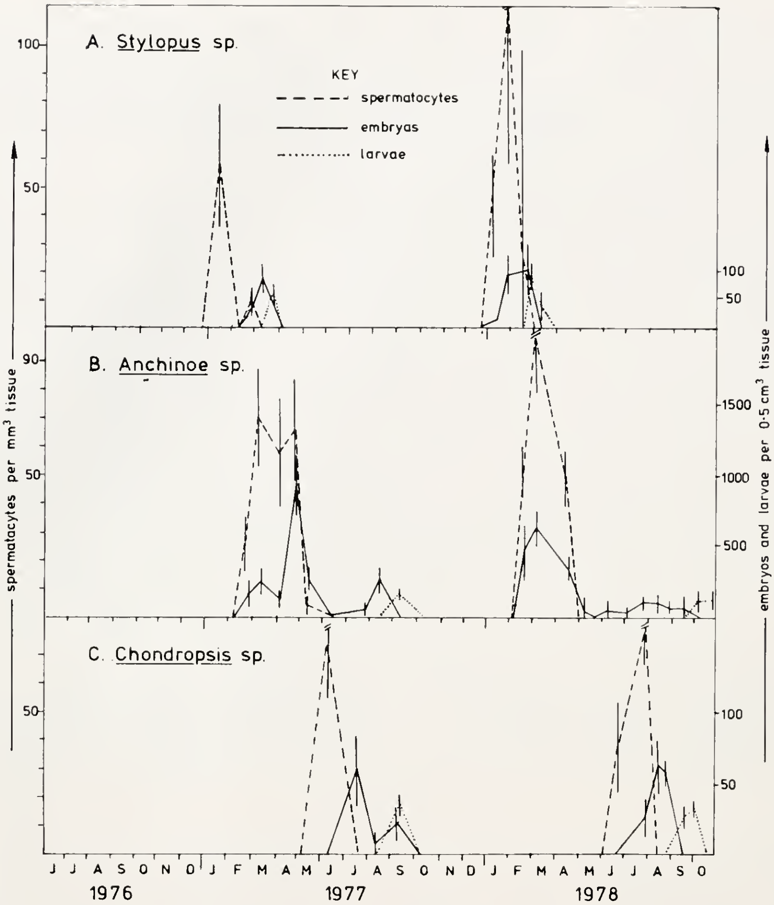

FIGURE 1. Abundance of reproductive elements in three species of thinly encrusting sponge. The data is derived from marked individuals that were continuously sampled. Vertical lines in the figures indicate plus or minus one standard error of the mean.

productive characteristics to the previously described *Stylopus* sp., both reproducing over the same summer months and producing similarly colored and shaped larvae. All six regularly sampled *Stylopus* sp. (pink) reproduced in both years of the study, but in the larger population samples only 39% (N = 41) reproduced in 1977 and in the following year, 67% (N = 70). All sizes of patches of this species were involved in reproduction. In some patches there were two episodes of spermatogenesis, with up to 100 spermatocytes per mm^3 tissue. Then embryos appeared in numbers of up to 0.2 per mm^3 tissue. Larvae emerged from single patches over a period of approximately 2 months and were released at a rate of 0.6–2 per cm^3 tissue per day. Larvae were 240 by 210 μm in size and covered with cilia except for the posterior pole, which was bare.

Anchinoe sp. This pale yellow sponge was uncommon in "beach canyon" and usually occurred as small patches in rock crevices. The reproductive period of *Anchinoe* sp. extended over most of the year, from February to November, and in both years of the study all the regularly sampled sponges except one reproduced. This latter sponge was a very small patch and produced sperm only in 1978. In the first year of the study at least 60% ($N = 15$) of the total population reproduced, in the second year at least 73% ($N = 15$) were active.

Anchinoe sp. is a simultaneous hermaphrodite which incubates its larvae. Sperm were present for the first 4 months only. Fertilized oocytes then developed for a period of 4 or 5 months and ciliated larvae finally appeared in the last 3 months (Fig. 1B). The reproductive products were located throughout the body of the sponge, spermatocytes in numbers of up to 100 per mm^3 and the very small embryos in numbers of up to 2 per mm^3. The pale yellow larvae were 179 by 146 μm in size and released at a rate of 5.4–20 per cm^3 tissue per day.

Chondropsis sp. The fragmented patches of this sandy sponge were abundant along the base of the "beach canyon" walls. *Chondropsis* sp. reproduced from early June to late September and all the regularly sampled sponges reproduced each year although in the second year all except one were extensively damaged by a violent storm in July. At least 36% ($N = 30$) of the larger sample reproduced in 1977 but only 25% ($N = 25$) reproduced in 1978.

Chondropsis sp. is a simultaneous hermaphrodite that incubates its larvae. Sperm were produced in the first few weeks of the reproductive period. Then embryos were found for the next 4 months, with ciliated larvae appearing in the last month. Spermatocytes in numbers of up to 70 per mm^3 and embryos in numbers of 0.1 per mm^3 tissue were located in patches throughout the mass of the sponge (Fig. 1C). The yellow larvae were released at a rate of 0.2–3 per cm^3 tissue per day and were shaped like flattened discs (as in *Halisarca*, Chen, 1976), 1.6 mm in diameter.

Reproductive patterns in discrete tetractinomorphs

Ancorina alata. This massive gray sponge could grow to a size of 2 square meters but was uncommon in the "sponge garden." *Ancorina alata* reproduced from early February to early June. All six continuously sampled individuals reproduced in both years of the study but in the larger population sample only 40% ($N = 60$) of sponges were reproductively active in the first year and 53% ($N = 28$) in the second year. *A. alata* is apparently gonochoric and oviparous. Over 2 years the sexual identity of each of the regularly sampled sponges was maintained and in each sponge there were frequently two cycles of sperm and oocyte production during the reproductive period (Fig. 2A). At the end of each cycle gametes were released over a week. The ratio of males to females was 1 : 5.3, the majority of the reproductive population thus being female. An examination of sections taken from various parts of the bodies of single sponges showed that the entire sponge was involved in production of only one type of gamete. In male sponges spermatocytes were located near the gray surface tissue in numbers of up to 1000 per mm^3 and in female sponges oocytes were located in the white body tissues in low numbers; about 50 per mm^3.

Polymastia sp. Polymastia sp. is a bright yellow spherical sponge abundant in the "sponge garden" with individuals up to 20 cm in diameter occurring singly or in groups of three or more. The beginning of the reproductive period of *Polymastia* sp. shifted over the 2-year period from August in 1976 to January in 1978. But both periods ended in May. In both periods all of the continuously sampled sponges reproduced. In the 1978 reproductive period 96% ($N = 156$) of sponges reproduced while in the pervious summer 56% ($N = 50$) were recorded

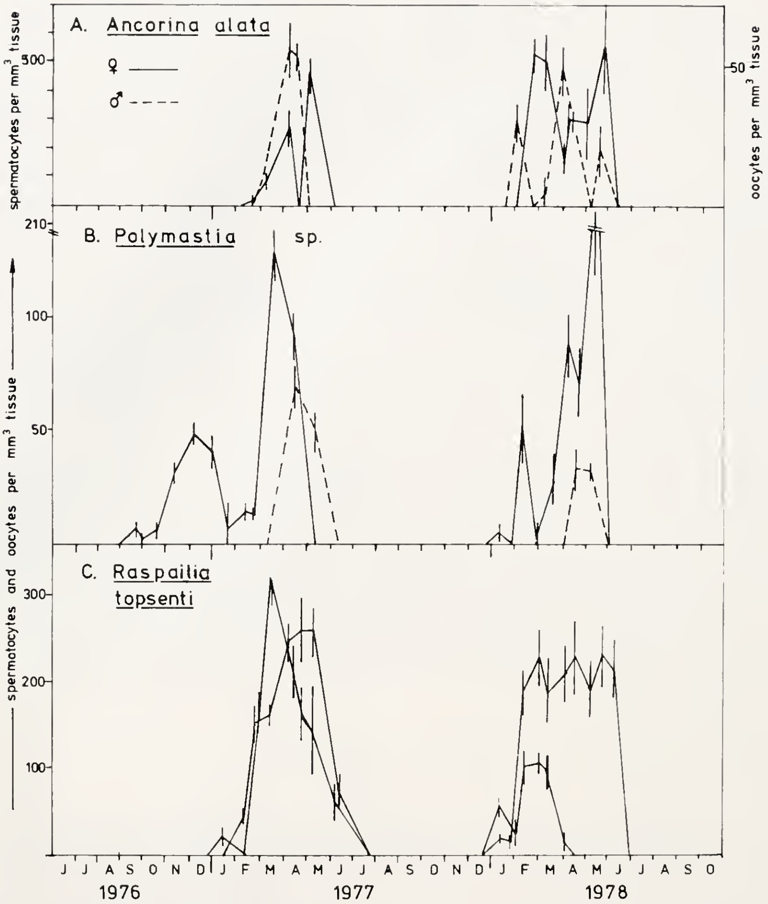

FIGURE 2. Abundance of reproductive elements in three species of discrete sponge. In the graphs of *Ancorina alata* and *Polymastia* sp. the results from one female and one male are shown while for *Raspailia topsenti* that of two females is shown. Vertical lines represent plus or minus one standard error of the mean.

as active. This latter percentage, however, was not taken at the end of the reproductive period and therefore does not include males.

Polymastia sp. is oviparous and apparently gonochoric. Regularly sampled individuals maintained their sexual identity over 2 years. The sex ratio of males to females was 1 : 1.09. Oocytes gradually increased in number over 6 months. Then sperm were produced in the last month of the reproductive period and both gametes released in the last week. Oocytes were always found in groups of two or three and occurred in numbers of up to 130 per mm³ tissue, while sperm appeared to fill large areas of the sponge body though the number of cysts was low: about 60 per mm³ tissue (Fig. 2B). Oocytes were extruded in adhesive masses containing 70 or more elements. Similar adhesive substances were noted by Borojević (1967) around the eggs of *P. robusta.*

Polymastia sp. also produced asexual buds from December to February in 1975–1976 and 1976–1977 but none over the summer of 1977–1978. Buds were approximately 5 mm in diameter and a single large *Polymastia* sp. might have as many as 17 buds at one time, which could be released at a rate of 0.2–4.9 per week per 100 cm^2 surface tissue.

Polymastia hirsuta. This maroon-colored spherical sponge, which may grow up to 15 cm in diameter, was uncommon in the "sponge garden." *Polymastia hirsuta* reproduced over the winter months from late May or June to July or October. In 1976 the regularly sampled sponges produced only oocytes, so in the following year three extra sponges were sampled for spermatocytes. One of these produced sperm. However, two sponges from the original group did not reproduce in this second year. The same pattern was repeated in 1978. Population samples taken in 1977 showed that at least 48% $(N = 50)$ of sponges were active and in 1978 at least 80% $(N = 30)$.

Polymastia hirsuta is apparently gonochoric and oviparous. The regularly sampled reproducing individuals maintained their sexual identity over the 3-year study period. Oocytes were accumulated over 4 months and sperm produced only in the last month of the reproductive period. Then both gametes were released over 2 weeks. Oocytes and spermatocytes were found throughout the sponge body in numbers of up to 30 per mm^3 tissue. The ratio of males to females was 1 : 1.99.

Aaptos aaptos. This abundant species is a small brown globe sponge supported on a thick pedicel, up to 10 cm in height and with a head diameter of up to 8 cm. The reproductive period of *Aaptos aaptos* was short—from September to October. As none of the continuously sampled sponges reproduced in 1976 a further sample of six individuals were also followed in the next year. Out of the new series one sponge reproduced and again was the only one to reproduce in 1978. In both years this sponge produced oocytes. In the population samples only 22.6% $(N = 92)$ of individuals were active in 1977 and 36% $(N = 30)$ in 1978. In the "sponge garden" only females were active in 1977, but at Outer Waterfall Reef (5 km from the "sponge garden") in the same year the ratio of males to females was 1:8.3. In 1978, males were sexually active in the "sponge garden" in a ratio of 1 : 1.8, males to females. These data and the evidence from the single regularly sampled sponge that reproduced suggest that *A. aaptos* is gonochoric. However, further sampling of marked individuals is necessary to establish the form of sexuality in this species. Sarà (1961) also suggests that this oviparous sponge is gonochoric. In the present study oocytes were found in numbers of up to 150 per mm^3 tissue and spermatocytes, often in groups, in numbers of up to 170 per mm^3 tissue.

Aaptos aaptos also produced asexual buds from February to April in 1977 but not in 1978. Buds were 2.0–5.0 mm in diameter and produced by 27% of the population at a rate of two per sponge per month.

Polymastia sp. (yellow). This small yellow sponge was the most abundant species of sponge in the "sponge garden" and consisted of a thin base from which arose slender oscule turrets up to 3 cm in height. Sexual products were found in the population in only three instances, once in April, 1977, and twice in January, 1978. Only oocytes were found and these occurred in the basal tissues in numbers of up to 220 per mm^3 tissue.

The major form of reproduction in *Polymastia* sp. (yellow) was the almost year-round production of small buds, which were produced from the tips of the fingers, sometimes in long beads that included the entire oscule turret. Buds were 1.0 mm in diameter and released at a rate of one per finger per month. Only

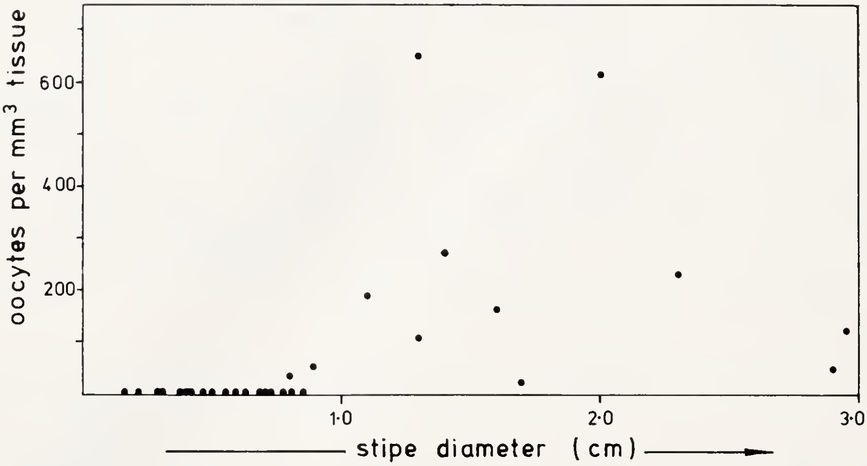

FIGURE 3. Relationship of reproductive activity to size in *Raspailia topsenti.*

13% of the regularly monitored sponges ($N = 30$) produced buds and the mean number of buds released was slightly higher in summer.

Raspailia topsenti. Small individuals of this abundant orange finger sponge consist of a single finger. Larger sponges may have as many as 60 fingers and grow to 30 cm in height. The reproductive period of *Raspailia topsenti* extended from the first weeks of January to the beginning of June. In 1977 four sponges of the regularly sampled group reproduced; in 1978 five of these reproduced. In the first year at least 34% ($N = 35$) of the population reproduced, in the second year 45% ($N = 31$) reproduced. No spermatocytes were found in the populations of this species at the "sponge garden" or at Outer Waterfall Reef. Oocytes were found in numbers of 320 per mm^3 in the fingers of the sponges (Fig. 2C). No sexual products were found in the pedicel or base tissues. There appeared to be a minimum size for the onset of reproductive ability in *R. topsenti,* as sponges of only one or two fingers and a pedicel diameter of less than 0.8 cm did not reproduce. Above this minimum size no relationship was found between size and the number of oocytes present (Fig. 3). After 6 months the oocytes disintegrated in a manner similar to that described by Gilbert (1974). Related species *Axinella damicornis* and *A. verrucosa* were found to be gonochoric and oviparous (Siribelli, 1962).

Settlement and recruitment of sponge larvae

The viviparous larvae of the thinly encrusting sponges took from 1–4 days to settle, while the oviparous larvae of *Polymastia* sp. took from 15–20 days to settle. All larvae crawled the entire time of their mobile lives, unlike many intertidal species which swim for various periods (Bergquist *et al.*, 1970). The viviparous larvae did not travel far after release and large sponge patches could be observed surrounded by settled larvae.

The settlement and recruitment of the larvae from the thinly encrusting sponges in "beach canyon" is recorded in Figure 4. The initial settlement of larvae from *Stylopus* sp. and *Stylopus* sp. (pink) is considered together as they were indistinguishable at that stage. Although large numbers of larvae settled on all monitored surfaces, successful recruitment occurred only on the natural crustose coralline surface. Only two *Stylopus* sp. and five *Stylopus* sp. (pink) survived over the 2 years. *Chondropsis* sp. also settled in large numbers but only 11 survived over the 2 years, again on natural surfaces. The larvae of *Anchinoe* sp. did not settle on any monitored surfaces, perhaps because no monitored areas were near patches of this species.

The recruitment rate of discrete sponges into the "sponge garden" was very low. *Polymastia* sp. (yellow) had the highest recruitment rate, at 0.6 per 0.0625 m^2 area natural habitat per year. Few individuals were recruited into the populations of the other species. Only one new individual each of *Polymastia* sp. and *Polymastia hirsuta* were recruited into the natural habitat. Unattached buds from *Polymastia* sp. could be observed especially over summer but none attached themselves to monitored surfaces.

DISCUSSION

The reproductive patterns of the 10 subtidal species of sponge investigated here show several general features. Two basic patterns were evident: the thinly encrusting ceractinomorphs, which were hermaphrodites producing large viviparous larvae; and the discrete tetractinomorphs, which were gonochoric over

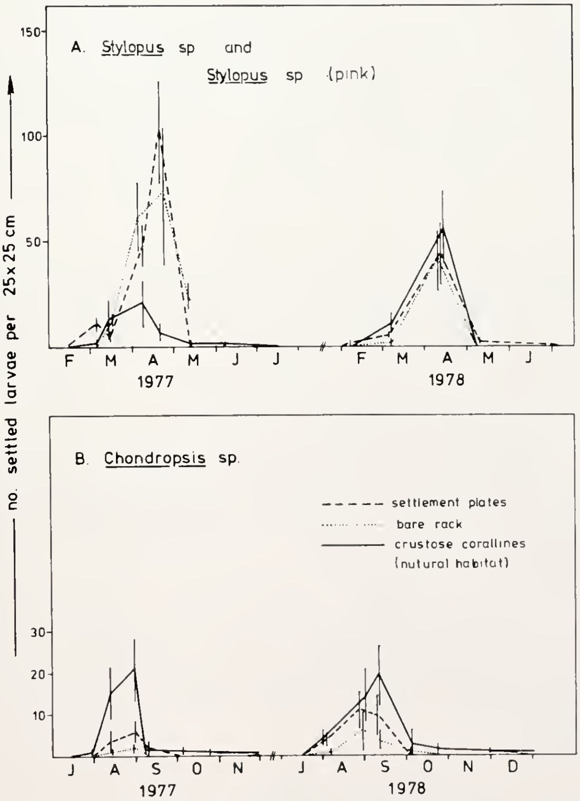

FIGURE 4. Settlement of the larave of *Stylopus* sp., *Stylopus* sp. (pink) and *Chondropsis* sp. on various surfaces. Vertical lines represent plus or minus one standard error of the mean.

2 years and apparently oviparous. Although the information collected is suggestive of the latter form of sexuality in *Raspailia topsenti, Aaptos aaptos,* and *Polymastia* sp. (yellow) further data are necessary to establish this conclusively. However, in general these data support the taxonomic division of the Demospongiae proposed by Lévi (1957) into the viviparous subclass Ceractinomorpha and the oviparous Tetractinomorpha.

The potential reproductive output of each viviparous species was different and some relationship of this to the abundance of the species was indicated. The small uncommon species *Anchinoe* sp. (also *Hymedesmia* sp. [red] and *Microciona* sp.; unpublished data) produced large numbers of larvae while the larger, spreading, abundant species such as *Stylopus* sp. and *S.* sp. (pink) produced fewer larvae. *Chondropsis* sp., although uncommon in the "beach canyon" area, produced few but much larger larvae than any of the other species.

In the reproductive patterns of the discrete species there appeared to be a relationship between length of reproductive period and release of gametes. Sponges such as *Ancorina alata* and *Aaptos aaptos* had short reproductive periods in which sperm and oocytes were produced simultaneously. In *Polymastia* sp. and *Polymastia hirsuta* oocytes were gradually accumulated over a long period. Then sperm were produced at the end of the reproductive period and released simultaneously with oocytes. In the discrete sponge populations there were few males. The males of *Aaptos aaptos* in the "sponge garden" were reproductively active in only 1 year of the study, suggesting that some of the non-reproductive members of the population are males. Thus the apparent sex ratio of the species can change each year. However, in general it appears that in sponge species there is usually a higher percentage of females reproductively active in the population than males (Hogg, 1967; Fell, 1976b; Johnson, 1978).

In both years of the study a percentage of each species population did not reproduce sexually. This non-reproductive percentage was not related to the sponges' ages, except in the case of *Raspailia topsenti* and *Aaptos aaptos,* but may have been related to temperature changes. In the second year of the study higher water temperatures were recorded than in the previous year and during this period a greater percentage of the population of each species was reproductively active. Such changes may have been due to temperature-related differences in food levels. Johnson (1978) found that habitat also affected the number of individuals reproducing in any year.

The different reproductive strategies appeared to be related to the level of disturbance the sponges normally encountered. The two sponge communities studied were subject to different intensities of disturbance. The thinly encrusting species in "beach canyon" were subject to the highest intensity of disturbance not only from urchins, which grazed 5% of the surface per year (A. L. Ayling, 1978a), but from surge produced by storms. An abundant leatherjacket fish (*Parika scaber*) also removed newly settled larvae (A. M. Ayling, 1976). In the "sponge garden" the overlying sediment made it impossible for urchins to graze or leatherjackets to graze effectively. Disturbance in this area was low and the only predators were opistobranchs, which were rare and which specialized on a few species of sponge.

The reproductive strategy of the thinly encrusting sponges living in the area of highest disturbance was to produce large larvae capable of rapid settlement and growth to sizes capable of withstanding grazing. Sponges that had reached 0.5 cm in diameter could be almost completely scraped off the substratum but still be able to reoccupy the lost space (A. L. Ayling, 1978a). Stimson (1978) has suggested that viviparous planulae of corals are adaptations for rapid settlement in fast-moving waters. The oviparous larvae of discrete sponges, on the other hand, were up to a 100 times more numerous (as determined from the potential output inside the sponge) than the viviparous larvae of the thinly encrusting species. But they were small and it appears that they may also crawl for long periods of time (Borojević, 1967; Bergquist *et al.*, 1970).

On release, subtidal viviparous larvae did not disperse widely but settled close to the parent sponges. The dispersion patterns of the discrete sponges (A. L. Ayling, 1978a) suggests that this may also be true of their larvae, but further work is needed. Such aggregated dispersion patterns produced by the settling larvae of subtidal sponges suggests that more detailed documentation of settlement patterns is needed than that currently obtained from a few randomly placed settlement plates or shells.

Settlement of larvae occurred on all surfaces but recruitment was most successful in the natural habitat. Sutherland and Karlson (1977) found that sponge larvae were common invaders of "mature" assemblages in epibenthic communities. Recruitment of sponge larvae on their ceramic tiles was very unpredictable. In the present study subtidal sponges reproduced regularly each year but the number of larvae surviving was unpredictable and generally low. In *Polymastia* sp. (yellow), however, the continuous production of buds ensured a predictable but low recruitment of individuals each year.

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SUMMARY

1. The patterns of sexuality and asexual reproduction in 10 species of subtidal Demospongiae were investigated by following reproductive activity in particular individuals and in the populations of each species over a 2-year period. Potential reproductive output was measured and related to recruitment rates recorded from natural and artificial surfaces.

2. The ceractinomorph sponges were found to be all hermaphrodites producing viviparous larvae while most of the tetractinomorph sponges were found to be gonochoric over the period of study and apparently oviparous. In the tetractinomorph sponge populations there was usually a majority of females and in two species no males were found. Three of the tetractinomorph species also produced buds.

3. Viviparous larvae are thought to be adaptations to habitats with high levels of disturbance.

4. Recruitment in both groups of sponges was low and unpredictable except in *Polymastia* sp. (yellow) which produced buds all year round and had predictable recruitment of low numbers of individuals. Recruitment occurred predominantly on natural surfaces.

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THE EFFECTS OF STARVATION ON PHOTOTAXIS AND SWIMMING OF LARVAE OF THE CRAB *RHITHROPANOPEUS HARRISII*

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The phototactic response pattern of the zoeal larval stages of crabs, which was described for *Rhithropanopeus harrisii* by Forward and Costlow (1974) and for various other light-adapted crabs by Forward (1977), is known to be altered by changes in environmental factors. Shifts in phototactic response have been described for *R. harrisii* larvae under conditions of altered salinity (Latz and Forward, 1977) and temperature (Ott and Forward, 1976), and for other species of crab larvae in altered pressure (Rice, 1964). It is likely that conditions in the internal environment also can influence phototactic behavior. Among these internal modifiers could be the nutritional condition of the larvae.

In other crustacean zooplankton, including larval forms, feeding or the presence of food has been reported to affect photobehavior. In *Daphnia magna*, Clarke (1932) found that positive phototaxis was minimal in media containing an abundance of food, but this effect could have been produced by the external medium. Lucas (1936) showed that the copepod *Eurytemora hirundoides* and the mysid *Neomysis* swam down, away from an overhead light, when in concentrated phytoplankton suspensions. On the other hand, Bainbridge (1953) reported that *Calanus finmarchicus* swam upward in overhead illumination when in the presence of phytoplankton. In the work of both Lucas (1936) and Bainbridge (1953), dark controls were not performed, so it is possible that the observed changes in vertical movement were not caused by a change in phototaxis. The most convincing work has been done with crustacean larvae. Singarajah *et al.* (1967) showed that starved stage-II nauplii of the barnacles *Elminius modestus* and *Balanus balanoides* have enhanced positive phototaxis and reduced negative phototaxis when compared to fed nauplii. The differences were most pronounced when the experiments were done in the filtered media of algal cultures. Zoea larvae of the anomuran crab *Emerita analoga* also are more positively phototactic and less negatively phototactic when starved (Burton, 1979).

This paper describes experiments to determine the effects of feeding on phototaxis and swimming of zoea larvae of the crab *Rhithropanopeus harrisii* (Gould). Starved early-stage larvae showed increased positive phototaxis and in some cases reduced negative phototaxis. The swimming speeds in all stages were generally lower in starved larvae.

MATERIALS AND METHODS

Larvae for all experiments were obtained from ovigerous specimens of *Rhithropanopeus harrisii* (Gould) collected from the Neuse River in eastern North Carolina. Crabs were maintained in filtered sea water at 25° C and 20‰ salinity and in a 12:12 light:dark cycle. Upon hatching, larvae were maintained in the same conditions as the female crabs in large finger bowls, and were transferred daily to clean sea water and fed newly hatched *Artemia salina* nauplii until used in experiments.

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Phototactic behavior was monitored by placing larvae in a quartz cuvette and viewing them through a stereomicroscope coupled to a closed-circuit television system (described in detail by Forward, 1974). All experiments were begun 5 to 7 hr after the onset of illumination of the light:dark culture cycle. Viewing illumination was filtered to 802 nm with an interference filter (Optics Technology, Inc.; half band pass, 39 nm). This wavelength does not affect phototactic behavior (Forward and Costlow, 1974). Stimulus illumination was provided in the horizontal plane by a LeBelle slide projector with a 300 W tungsten bulb. Horizontal stimulation permits separation of phototactic responses from geotactic responses. Light was filtered by two hot mirrors (Baird Atomic) and a Corning #1–75 filter to reduce heat, and filtered to 500 nm by an interference filter (Ditric Optics, half band pass 7.4 nm). This wavelength was selected because it corresponds to the sensitivity maximum of the larvae (Forward and Costlow, 1974). Intensity of the stimulus was regulated by neutral density filters and was measured with a YSI model 65 radiometer. The duration of the stimulus was set at 2 sec and was controlled by an electromagnetic shutter (Uniblitz model 225XOROX5, with a model 310 drive unit).

Responses were recorded on video tape and analyzed as reported previously (Forward and Costlow, 1974). Positive phototaxis was defined as movement toward the stimulus source (± 15°), while negative phototaxis was movement 180° to the stimulus source (± 15°). Swimming direction and speeds for the dark control were determined immediately before the onset of a light stimulus. Swimming speeds were determined by measuring the curvilinear distance traveled in 0.5 sec. Results of these analyses were compared using standard analysis of variance techniques (Sokal and Rohlf, 1969).

In studies of phototaxis, all larvae were light-adapted under room lights plus a 60 W incandescent light for at least 1 hr prior to experimentation. Larvae were pipetted into the experimental cuvette and were used only once on any day. One min was allowed in total darkness on the microscope stage before the first stimulation; stimuli were then spaced at 15-sec intervals. The procedure used varied with each experiment and is described below.

Experiment 1—Effects of feeding and prey medium upon phototaxis

Larvae of stages I (4 broods) and III (3 broods) were used. Experimental stage-I larvae from each brood were fed for 1 day after hatching and then divided into two equal portions: the first was fed for a second day (fed group) while the second received no food (starved group). At the end of this second day, larvae were again changed to fresh 20‰ sea water and then each group was further subdivided into 2 groups; one placed in fresh filtered sea water of 20‰ and the other in a mixture of 20‰ filtered sea water mixed in equal parts with water in which *Artemia salina* nauplii had been hatched and reared for 24 hr, filtered and adjusted to 20‰ with distilled water. Thus there were 4 subgroups to be tested: fed—sea water; fed—"*Artemia* medium"; starved—sea water; and starved—"*Artemia* medium."

After 1 hr in the new medium, larvae in each subgroup were tested for phototaxis over the intensity range of 2.3×10^{-5} W/m^2 to 2.3 W/m^2 in 0.5 log unit steps. Each sample of larvae was stimulated at 4 intensities, either in ascending or descending order of intensity, and then discarded. Two larval samples from each brood were tested at each intensity. In order to study the effect of stage on the relationship between nutritional state and phototaxis, stage-III larvae were treated similarly to stage I, except that they were reared normally until the first day after the molt to the third zoeal stage. They were then divided into fed and starved groups and treated identically to the stage-I larvae.

Experiment 2—Ontogeny of effect of feeding upon phototaxis and swimming

The results of experiment 1 indicated different responses of stage-I and stage-III larvae to lack of feeding. In order to investigate whether this was due to an ontogenetic effect or to a difference in amount of food reserves in various stages, all four zoeal stages were tested for their phototactic response under fed and starved conditions. Originally, it was planned that each successive stage would be starved for a greater amount of time. However, it was found that after 3 days of starvation, the later-stage larvae were not suitable for testing due to high mortality and inability to swim well. Thus the starvation times for each stage were as follows: stage I, 1 day; stage II, 2 days; stage III, 3 days; stage IV, 3 days. If larvae of each stage are fed after these starvation times, they will generally show good recovery and low subsequent mortality. The rearing regime for stage I was identical to that used in experiment 1. For the later stages, on the morning following the molt to the desired stage the larvae were subdivided into fed and starved groups. Thereafter, each group was changed daily into clean water, tested, and the fed group given fresh *Artemia salina* nauplii. On the final day of testing, each group was further subdivided into sea water and "*Artemia* medium" subgroups, as in experiment 1, before testing. Because fed larvae typically molted to the next stage after 2 days, it was not always possible to test fed larvae on the second and later days in each stage.

Usually, larvae from three broods were tested at each stage, but four broods were used for stage II, day 1; and two broods used for stage III, day 3. Larvae were tested for positive phototaxis at an intensity of 6.0×10^{-2} W/m^2 and for negative phototaxis at an intensity of 6.0×10^{-4} W/m^2. These intensities were selected for evoking the desired response by reference to the results of experiment 1 and to the work of Forward and Costlow (1974). Each sample was tested only once at each intensity and then returned to clean sea water or discarded. Three samples were tested at each intensity for each brood. Swimming speeds were measured during random swimming, positive phototaxis and negative phototaxis.

Experiment 3—Ontogeny of feeding rate

In order to investigate the effect of time of day and of stage on larval feeding, 18 larvae from each of two broods were isolated within 2 hr of hatching into wells of glass spotting plates (average volume, 1.4 ml), and maintained until the molt to the first crab stage. Conditions were as for other experiments; that is, 25° C, 20‰ salinity, and a 12 : 12 L : D cycle. At the end of the light period and the end of the dark period they were observed under a dissecting microscope and the number of individual *Artemia salina* nauplii consumed in the preceding

TABLE I

Results of an analysis of variance on the effects of light intensity at 500 nm, nutritional state (fed vs. starved), and medium (20‰ sea water vs. "Artemia medium") on positive and negative phototaxis by first and third stage larvae. d.f. = degrees of freedom.

Source of variation	Zoea I			Zoea III		
	d.f.	Positive phototaxis (F value)	Negative phototaxis (F value)	d.f.	Positive phototaxis (F value)	Negative phototaxis (F value)
Light intensity	10, 132	56.05***	4.57***	10, 88	10.58***	2.71**
Nutritional state	1, 132	39.43***	4.22*	1, 88	0.70	0.02
Medium	1, 132	2.01	0.05	1, 88	0.26	0.65
Intensity × nutritional state	10, 132	0.79	1.44	10, 88	0.50	1.25
Medium × nutritional state	1, 132	0.48	0.21	1, 88	0.03	0.77
Intensity × medium	10, 132	0.51	0.56	10, 88	0.37	0.72
Intensity × nutritional state × medium	10, 132	0.33	0.71	10, 88	0.68	1.03
Error	132			88		
Total	175			131		

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

3- or 12-hr interval was noted. Observations during the dark period were made using a microscope lamp interference filtered to 700 nm, a wavelength to which the larvae are very insensitive, to provide as little visible light to the larvae as possible.

During the first $2\frac{1}{2}$ days, the number of newly hatched brine shrimp nauplii was restored at the end of each 3-hr period to the number appropriate for the stage of that individual larva (see below). After each 12 hr throughout the experiment, the larvae were changed to fresh 20‰ sea water containing a number of newly hatched nauplii which was greater than the number of nauplii which could be consumed in 12 hr. Ten nauplii were supplied to each stage-I larva, 25 to stages II through IV, and 30 to each megalopa. The total number of nauplii consumed by each larva, the number consumed in each larval stage, and the difference between the number eaten at night and during the day were thus determined.

RESULTS

Effect of starvation on responses to a range of light intensities

In experiment 1, stage-I and stage-III larvae were tested for positive and negative phototaxis over a range of light intensities. Tests were performed in both filtered sea water and in "*Artemia* medium." The results of the experiments for each stage were analyzed by a 3-way ANOVA, with the main effects being light intensity, nutritional state, and medium (Table I). Both light intensity and nutritional state had significant effects on phototaxis in stage I, while in stage III only light intensity was significant. In each stage, the same main effects had statistically significant influences on both positive and negative phototaxis.

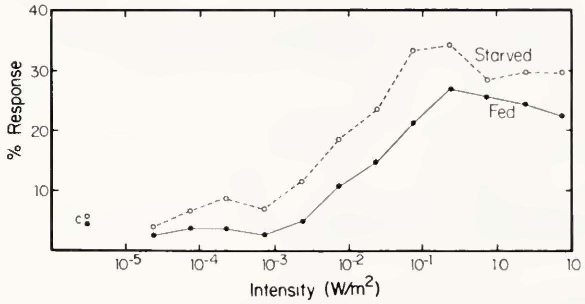

FIGURE 1. Per cent response (ordinate) of positively phototactic fed (closed circles, solid line) and starved (open circles, dashed line) stage-I zoea larvae to various intensities of 500 nm light (abscissa). The average sample size for each point is 366 larvae (fed) or 340 larvae (starved). C indicates the control level of response.

Since there was no significant effect of medium, data for all stage-I larvae were combined for each feeding condition and light intensity. The combined data show that in stage I, starved larvae were consistently more positively phototactic than fed larvae at all intensities tested (Fig. 1). The effect of light intensity was the usual one, leading to increased response at higher light intensities (Forward and Costlow, 1974). For negative phototaxis, starved stage-I larvae generally had a slightly reduced response compared to fed larvae (Fig. 2), particularly at the high intensities. Again, the pattern with respect to intensity for both starved and fed larvae was similar to that found by Forward and Costlow (1974) for negative phototaxis; *i.e.*, highest response to relatively low intensities (10^{-3}–10^{-4} W/m^2) for light-adapted larvae.

For stage-III larvae, both positive and negative phototaxis were affected significantly only by light intensity level, so the data for both nutritional states and both media were combined at each intensity. The overall pattern for both positive and negative phototaxis was the typical one described by Forward and Costlow (1974).

Effects of increasing length of starvation on phototactic response

Although stage-III larvae did not show altered phototaxis after 1 day of starvation, this could be because they have food reserves adequate to maintain

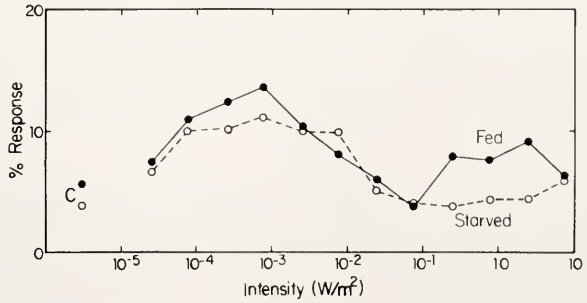

FIGURE 2. Per cent response (ordinate) of negatively phototactic fed (closed circles, solid line) and starved (open circles, dashed line) stage-I zoea larvae to various intensities of 500 nm light (abscissa). The average sample size for each point is 366 larvae (fed) or 340 larvae (starved). C indicates the control level of response.

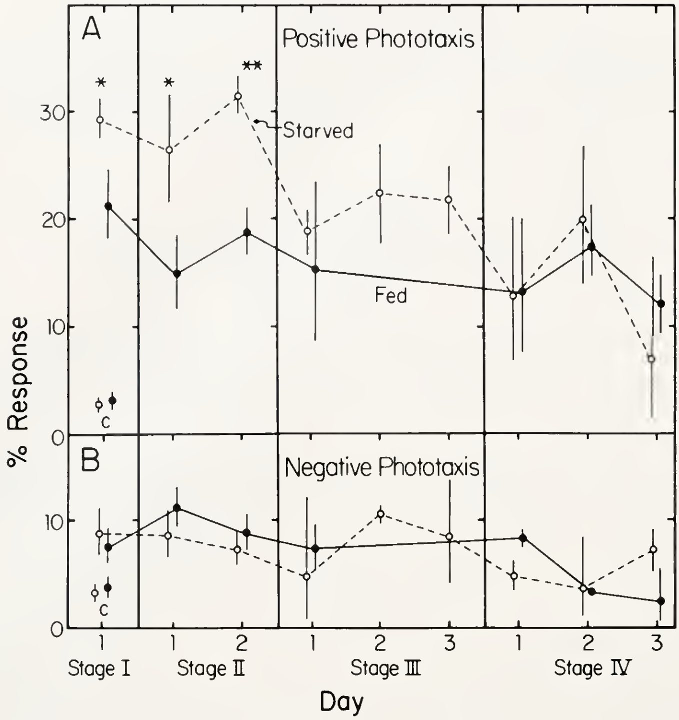

FIGURE 3. Per cent phototaxis (ordinate) vs. stage and day (abscissa) for the zoeal stages of crab larvae fed (closed circles, solid lines) or starved (open circles, dashed lines) for various amounts of time. Each point shows the standard error for, usually, 3 broods of larvae (see text). Positive phototaxis is shown in A, negative phototaxis in B. Significantly different responses between fed and starved larvae (one-tailed t-test) are indicated by asterisks above the significantly different pair (* $P < 0.5$; ** $P < 0.01$). C indicates the control level of response. The sample size for each point ranged from 46 (positive phototaxis, starved, stage III, day 3) to 357 (positive phototaxis, fed, stage I, day 1).

them in good nutritional condition for longer than 1 day. According to this view, the behavior of the larger late-stage larvae would change at a slower rate with starvation time than that of earlier stages. To test this hypothesis, fed and starved larvae of each successive stage were tested for increasing numbers of days. On the final day of testing for each stage, the effect of medium on positive and negative phototaxis of fed and starved larvae was also tested. In no case was the effect of medium found to be significant ($P > 0.05$), so all data for larvae in sea water and "*Artemia* medium" were combined for portrayal and further analysis.

In stages I and II, positive phototaxis of starved larvae significantly exceeded that of fed larvae (Fig. 3). Although in stage III the mean level of positive phototaxis of starved larvae was always higher than that of fed larvae, there were no statistically significant differences between positively phototactic responses of starved and fed larvae in stages III and IV. Similarly, although positive phototaxis on the second day of starvation always exceeded that on the first day, no statistically significant trends were found in change of level of positive phototaxis with increasing starvation time.

With regard to negative phototaxis, there was no indication of a starvation effect at any time, not even in stage I. The results thus differ from those of experiment 1; this was probably because negative phototaxis was measured at only one intensity; note in Figure 1 that negative phototaxis was reduced the most at high light intensities.

Starvation and swimming speeds

The results of experiment 2 were analyzed for swimming speeds as well as phototaxis (Fig. 4). When swimming in the dark was being measured, starved

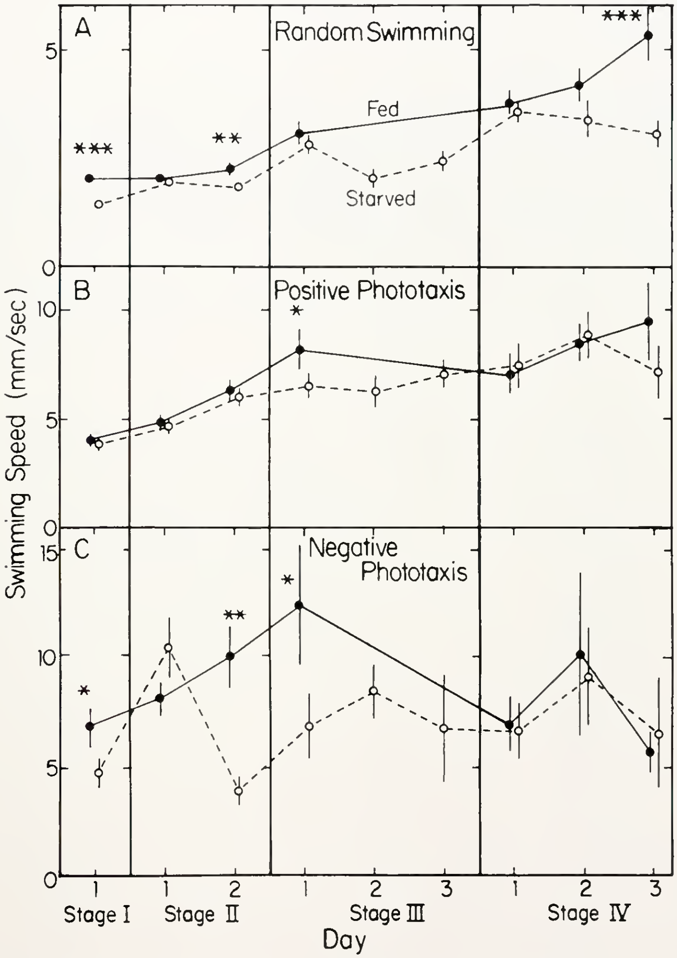

FIGURE 4. Swimming speeds in mm/sec (ordinate) of zoeal stages of crab larvae fed (closed circles, solid lines) or starved (open circles, dashed lines) for various amounts of time (abscissa). Each point shows the mean ± standard error for larvae in each condition. Random swimming (no stimulus) is shown in A, swimming in positive phototaxis in B, and swimming in negative phototaxis in C. Significant differences between fed and starved larvae (one-tailed t-test) are indicated by asterisks above the significantly different pair ($* P < 0.05$; $** P < 0.01$; $*** P < 0.001$). The sample size for each point depended upon the number of larvae responding in positive and negative phototaxis; it ranged from 43 (starved, stage IV, day 2) to 141 (starved, stage I, day 1) for random swimming; from 9 (starved, stage IV, day 3) to 78 (fed, stage I, day 1) for positive phototaxis; and from 3 (fed, stage IV, days 2 and 3) to 30 (fed, stage I, day 1) for negative phototaxis.

larvae always swam more slowly than fed larvae; the difference, as tested by a t-test (one-tailed) for each day, was significant for stage I, day 1; stage II, day 2; and stage IV, day 3. During positive phototaxis, swimming speeds of fed larvae again usually exceeded those of starved larvae, but the effect was only significant for larvae in stage III, day 1. Swimming speeds of fed larvae during negative phototaxis were also usually higher than those of starved larvae; the difference was significant for stage I, day 1; stage II, day 2; and stage III, day 1. There was little evidence in any of the conditions measured that speed of swimming in larvae decreased further as they were starved for longer periods; only once (stage II, negative phototaxis, $P < 0.001$) did swimming speed significantly decline as starvation progressed.

Time of day and larval feeding

The average number of individual *Artemia salina* nauplii consumed per 12-hr period (night or day) is shown in Figure 5. Only time periods which did not include a molt and only larvae which completed metamorphosis to the first crab stage (n = 32) were used in the analysis. The results of a 2-way ANOVA on

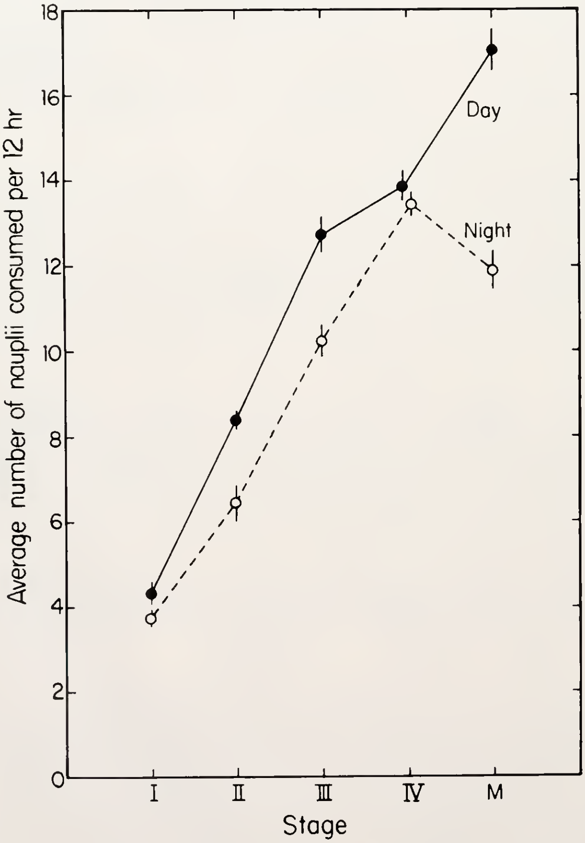

Figure 5. Average number of newly-hatched *Artemia salina* nauplii (ordinate) consumed by crab larvae at various stages (abscissa) during the day (closed circles, solid line) or night (open circles, dashed line). Each point shows the mean and standard error for 32 larvae.

TABLE II

Results of an analysis of variance on the effects of larval stage (zoea I through megalopa) and time of day (day vs. night) on the number of brine shrimp nauplii consumed in a 12-hr period. d.f = degrees of freedom.

F Values Source of variation	d.f.	F
State	4, 310	397.346***
Time of day	1, 310	86.8825***
Interaction	4, 310	13.9951***

*** $P < 0.001$.

this data are included in Table II; both of the main effects of stage and time of day were highly significant. The effect of stage was seen as a rapid increase in consumption of nauplii with each molt, while the time effect was a greater feeding rate during the day as compared to night.

The differences in consumption with time of day were somewhat different in the three earliest stages, with more nauplii eaten during the day, from stage IV, in which daytime and nighttime rates were very similar. The megalopa showed the greatest difference between daytime and nighttime rates, consuming 143% as many napulii during the day as during the night.

DISCUSSION

Early-stage (first and second zoea) *Rhithropanopeus harrisii* larvae showed enhanced positive phototaxis when they were starved for a day or more. This change in phototaxis was not seen in the later, third- and fourth-stage zoea larvae. Also, upon stimulation with high light intensities, stage-I larvae had a reduced level of negative phototaxis after starvation.

In contrast to some earlier studies of planktonic crustaceans and crustacean larvae (Bainbridge, 1953; Clark, 1932; Singarajah *et al.*, 1967), the nature of the test medium did not affect either phototaxis or swimming speeds. Larvae of *R. harrisii* are sensitive to the medium in which they are living, as they are very responsive to changes in salinity (Latz and Forward, 1977); and we have often noted that day-old larval cultures become more responsive to an incandescent light source following tranfer to clean 20‰ sea water. It is possible that they are also sensitive to exudates of some prey forms but not to those of *Artemia salina* nauplii, admittedly not a normal food item.

The general pattern of an increased level of positive phototaxis and/or a reduction in negative phototaxis in starved animals has now been demonstrated for a variety of planktonic crustaceans, particularly larval forms. Clarke (1932) noted that *Daphnia magna* was most photonegative when living in fresh cultures containing plenty of food; however, this could have been an effect of the medium. Singarajah *et al.* (1967) showed that nauplii of the barnacles *Eliminus modestus* and *Balanus balanoides* had both an increase in photopositive behavior and a decerase in photonegativity when they were starved, as compared to the fed controls. Unlike the situtation for *R. harrisii* larvae, these starved barnacle nauplii were even more photopositive when tested in media from the food cultures.

Among larvae of crabs, first-stage zoea larvae of the mole crab *Emerita analoga* became increasingly photopositive during starvation from 2 to 4 days; fed larvae, on the other hand, became more negatively phototactic over this period (Burton, 1979). Although the level of positive phototaxis of *R. harrisii* larvae increased on the second day of starvation in stages II, III, and IV, this increase was not statistically significant and the pattern of change in negative phototaxis is irregular. Thus, there is little evidence that increasing the duration of starvation potentiates its effect on phototactic behavior in *R. harrisi* larvae.

In *R. harrisii* larvae, the starvation effect is mostly an enhancement of positive phototaxis; negative phototaxis is affected much less than positive in stage I (the effect being seen mostly at higher light intensities) and not at all in the later stages. The change could be because of greater energy stores in the later stages; in fact, some stage-IV larvae starved for longer than 3 days successfully molted to the megalopa. Alternatively, the phototactic pattern could be more conservative in later stages; Latz and Forward (1977) have found that the change in phototactic pattern with changing salinity is more extreme in stage-I *R. harrisii* larvae than in stage IV.

The swimming of starved larvae was generally slower than that of well-fed larvae from the same brood. The difference in speed was most evident during negative phototaxis, when fed larvae reach their maximum speeds (Forward and Costlow, 1974). The amount of reduction of swimming speeds in starved larvae is generally quite constant with increased time of starvation in a given stage; only in stage II is there a significant change in swimming speed after the first day of starvation. This generally decreased activity level during starvation is probably a result of energy conservative mechanisms, and not deteriorative effects, as late-stage larvae will survive up to 3 days of starvation with good recovery on subsequent feeding.

Feeding rates in *R. harrisii* larvae are known to decline at concentrations of *Artemia salina* nauplii lower than 2 to 5 nauplii per ml (Welch and Sulkin, 1974), but the concentrations used in this study exceeded this level (minimum initial concentration: 7.1 nauplii per ml). Therefore, the larvae were probably consuming prey at a maximum rate. This is also suggested because many of the nauplii taken as prey were only partly eaten. Often only the yolk-rich posterior portion was consumed and the anterior portion with its swimming appendages discarded. This was perhaps partly because crab larvae usually consume nauplii prey from the abdomen forward, and when the nauplius is held in this position, its swimming appendages are immoblized. Sulkin (1975) and Sulkin and Norman (1976) have suggested that the lipid yolk material stored in the abdomen region of *Artemia* nauplii is important for development in crab larvae.

When placed in a light: dark cycle, *R. harrisii* larvae consume more food during the 12-hr light phase than during the equal period of dark. However, zoea larvae continue to take in from 43 to 49% of their total daily food during the night, and the megalopa consumes 41% of its daily intake at night. The fact that feeding activity continues to occur at night suggests that visual orientation to prey is not essential for feeding and that the slightly depressed nighttime feeding levels probably reflect a generally lowered feeding activity. Although crab larvae have been reported to show a directed strike behavior (Knudsen, 1960), they are thought not to use sight to localize prey (Gonor and Gonor, 1973). For example, larvae of *Uca pugilator* apparently locate and trap prey by a mechanical tail-lashing technique (Herrnkind, 1968). Since *R. harrisii* larvae feed less at night, the possibility of their photobehavior being altered by feeding state is strongest in the early part of the day, and at this time they could show the highest levels of positive phototaxis. This has not been tested, but any effect is probably very slight.

The overall effects of starvation on phototaxis of *R. harrisii* larvae will probably bring them nearer to the surface in a natural water column, where food is presumably more abundant. This would also tend to move them to other parts of the estuary, where more abundant food supplies might be encountered. But it would simultaneously increase the rate of their transport by currents toward the mouth of the estuary in which they are living. In addition, starved larvae will be much more vulnerable to visually oriented predation, and since their swimming speeds are reduced they will have less chance of escape. The reduction of negative phototaxis in stage I could also be important, since this would reduce the shadow response which is probably used to avoid drifting cnidarian and ctenophore predators (Forward, 1976, 1977). Because both the response level and the swimming speeds are reduced in stage-I larvae during negative phototaxis, they will be particularly susceptible to this type of predation. On the other hand, most of the reduction in negative phototaxis occurs at higher light intensities, but the shadow response is most active at quite low intensities (Forward, 1976), so this response will remain somewhat effective.

Once fed, the larvae of all stages will probably regain the response pattern typical of well-fed larvae and move down in the water column. This migration pattern would be very similar to that described for *Sagitta elegans* by Pearre (1973), in which the chaetognaths swam downward when satiated, but remained near the surface, particularly at night, when hungry. One important difference between *S. elegans* and *R. harrisii* larvae, however, is that in the chaeteognaths feeding occurs mainly at night, but the crab larvae consume most of their food during the day.

The phototactic response pattern of crab larvae is sensitively tuned to the environment. Changes in the external environment alter phototaxis, as shown for salinity (Latz and Forward, 1977) and for temperature (Ott and Forward, 1976). To these external influences can be added the internal modifier of nutritional state.

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SUMMARY

1. Stage-I zoea larvae became more positively phototactic and less negatively phototactic after 1 day of starvation. Stage-II larvae also had an increased positive phototaxis after 1 or 2 days of starvation, but their negative phototaxis was not changed. Later stages did not have altered phototaxis during starvation.

2. The swimming speeds of starved larvae were generally lower than those of fed larvae, but there was no further reduction in the swimming speed after the first day of starvation.

3. When kept in a 12: 12 light : dark cycle, larvae of all stages consumed more food during the day than at night, but feeding in the dark was still effective. Therefore, there probably is little effect of nutritional condition on phototaxis over the course of a day.

4. The altered phototaxis of starved larvae would move them higher in the water column, where food is presumably more abundant, and would also increase their opportunity to be carried into new regions of an estuary where they could encounter food. However, the reduced negative phototaxis and lowered swimming speeds of starved larvae probably would place them in increased risk of both predation and export from estuarine waters.

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TIDAL RHYTHMS OF ACTIVITY AND PHOTOTAXIS OF AN ESTUARINE CRAB LARVA

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In estuaries, the net flow of water is seaward. As a consequence, estuarine plankton continually risk export into adjacent coastal waters. This risk is especially severe among many of the relatively short lived planktonic larvae of benthic invertebrates, whose survival depends upon being in the estuary near a suitable adult habitat at metamorphosis.

Larvae of the estuarine crab *Rhithropanopeus harrisii* are retained in the estuary near the adult population throughout development (Cronin, 1979; Sandifer, 1973, 1975). Cronin (1979) studied the vertical distribution of *R. harrisii* larvae in an area having pronounced semi-diurnal tides and found that all larval stages usually occur near the depth of no net motion. Although this generally accounts for their retention near the parent population, larvae do not continually stay at the depth of no net motion. Rather, they migrate vertically around this depth. These movements are related to environmental cycles in light and dark, salinity and current speed (Cronin, 1979). Further laboratory studies indicate that the larvae have a pronounced endogenous tidal vertical migration pattern in which the maximum depth is reached around the time of low tide and minimum at high tide (Cronin and Forward, 1979).

The present study was initiated to investigate the behavioral basis of these vertical migration rhythms in *R. harrisii* larvae. Swimming speed and light oriented movement (phototaxis) were monitored in freshly caught larvae. These types of behavior were chosen because vertical movements may result from changes in activity, and phototaxis may contribute to daily migrations relative to the natural light: dark cycle. We found endogenous tidal rhythms in both swimming speed and phototaxis.

MATERIALS AND METHODS

Larvae from the crab *Rhithropanopeus harrisii* (Gould) were collected with a No. 2 plankton net in the Newport River estuary, North Carolina. All collections were during daytime high tides in water of about 16‰ salinity at depths of about 0.5 to 1 m. Larvae were taken rapidly to the laboratory, where stage III zoeae were sorted out for experimentation. This stage was used because it has a pronounced endogenous rhythm in tidal vertical migration (Cronin and Forward, 1979). Groups of larvae were placed in 11.4-cm-diameter finger bowls in water from the collection site, which was filtered to remove particles larger than 5 μ. Thereafter, the larvae were maintained under constant conditions of light (cool white fluorescent lamp; intensity of 19 W m^{-2}), salinity, and temperature (30° C ± 0.5°) in a Sherer controlled environmental chamber (model CEL 4-4). This temperature was used because water temperatures at the collection site ranged around 30° C.

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The rhythm in speed of swimming was measured on two occasions (beginning dates July 24 and 30, 1979). The time of tides had advanced about 4 hr from the first date to the second. In both cases, three groups of 50 larvae each were placed in the controlled environmental chamber. At 2-hr intervals, all of the larvae from each bowl were placed in a lucite cuvette on the stage of a dissecting microscope and observed by means of a closed-circuit television system (Forward, 1974a; Forward and Costlow, 1974). The microscope illumination was filtered to the infrared (IR) region (American Optical Co. Filter No. 775–752). The larvae are insensitive to wavelengths in this region (Forward and Costlow, 1974).

The exact procedure was to place the 50 larvae on the microscope stage for 1 min in darkness, and then record swimming on video tape for 30 to 45 sec. Larvae were returned to the controlled environmental chamber, placed in new water and given a small amount of *Artemia salina* nauplii. In this way the larvae were placed in new water and given new food every 2 hr. Newly hatched *A. salina* nauplii were obtained each morning and used throughout the day. This procedure maintained the larvae in a viable condition, as indicated by the fact that an average of 89% (SE = 1%) of the larvae were alive at the end of each experiment. Swimming speeds of 15 larvae chosen arbitrarily from each group were analyzed using methods previously described (Forward and Costlow, 1974).

Phototaxis was measured by light stimulation in the horizontal plane in order to avoid complications due to directional responses to gravity (geotaxis). The stimulus source was a slide projector having a 300 W incandescent bulb. The light was filtered by two hot mirrors (Baird Atomic) and a Corning No. 1–75 IR absorbing filter in order to remove heat and was then filtered to 500 nm with an interference filter (Optics Technology; half band pass 14 nm). The larvae are maximally sensitive to wavelengths in this region (Forward and Costlow, 1974). The light intensity was regulated by neutral density filters and was measured with a radiometer (EG and G, model 550).

The test chamber consisted of a rectangular lucite cuvette (15 × 3 × 3 cm), which was divided into five equal sections along the longitudinal axis. The sections were separated by thin partitions constructed so that all could be moved vertically in unison. For determining phototactic responsiveness, a group of animals was placed in the center section of the cuvette. The animals were left 1 min in darkness, the partitions separating the sections were raised, the animals were stimulated for 30 sec, and then the partitions were returned. The distribution of animals among the five sections was then recorded. Control responses were determined by following the same procedure, except that animals remained in darkness for the entire trial. For establishing phototactic response levels, animals located in the two sections nearest the light source were considered to display positive phototaxis, while those in the furthest two sections were considered negative.

There were two series of phototactic experiments. In the first, responsiveness to a range of stimulus intensities was measured shortly before high and low tides. Larvae were collected at high tide in the morning on two consecutive days, taken rapidly to the laboratory, sorted into seven groups of 10 larvae each, and maintained in a light-adapted state in water from the collection site, under the conditions described above in the constant environmental chamber. Beginning an average of 45 min before the subsequent low tide, phototactic responsiveness to a range of

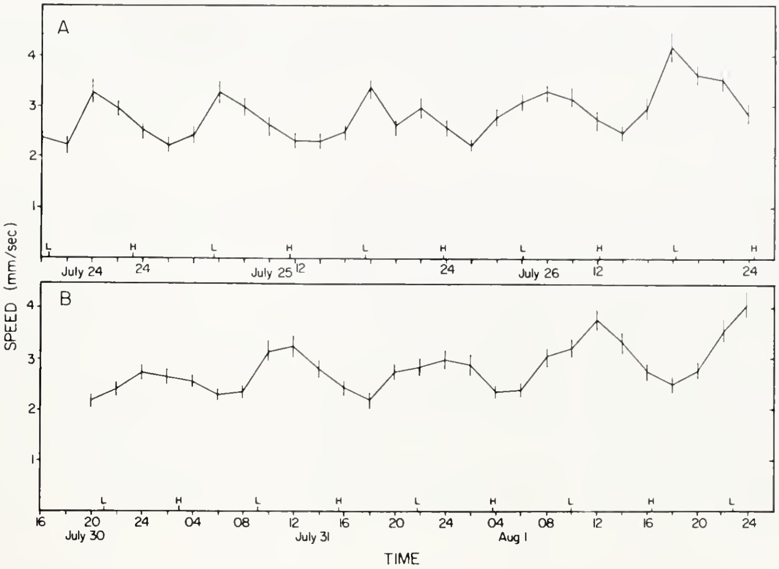

FIGURE 1. Rhythm in swimming speeds (ordinate) over time (abscissa) for larvae collected on July 24 (A) and July 30, 1979 (B). Mean speeds and standard error of 45 determinations are shown. L is time of low tide, and H shows high tide.

intensities was measured. Each group of larvae was tested at two different intensities. Larvae were then maintained in the environmental chamber until about 45 min before the next high tide, when photoresponsiveness was again tested. Tidal times were established by adjusting the NOAA tide table times for Pivers Island, North Carolina, to a predicted time at the collecting site in the Newport River estuary (+ 1.5 hr).

The second experimental series involved determining the rhythm in phototaxis. Larvae were collected on two occasions (August 6 and 15, 1979). Tidal times advanced about 8 hr between the two runs. Larvae were sorted into three groups of about 30 individuals each and placed under constant conditions. At 2-hr intervals, phototaxis by larvae in each bowl was tested using the described procedure. After phototactic responsiveness was tested, the larvae were placed in new water from the collection site and fed *A. salina* nauplii.

RESULTS

Rhythm in swimming speeds

The larvae have a clear endogenous tidal rhythm in swimming speeds (Fig. 1). In both trials (Fig. 1A, B) using the Fisher periodogram test (Fuller, 1976) the frequency nearest the tidal cycle is significant ($P < 0.05$). The speeds increase to a maximum within 3 hr after low tide, and then decrease to a minimum about 2 hr after high tide. This pattern is seen on both test dates and is related to tidal times. This is indicated by the fact that both the time of tides and of rhythmic swimming advance by 4 hr from trial 1 (Fig. 1A) to trial 2 (Fig. 1B).

Speeds vary between similar maximum and minimum values over about the first 36 hr. The final maximum values are much higher. This reflects the molt of most larvae to stage IV zoeae which swim faster than stage III (Forward and Costlow, 1974). Even with this molt, the rhythm continues under constant conditions for at least 54 hr. There is no indication of a diel component of the rhythm.

Phototaxis

Generally the larvae respond positively to high light intensities and negatively to low (Fig. 2), which is the typical pattern (Forward and Costlow, 1974). The curves of responsiveness to different light intensities suggest that phototaxis varies between high and low tide (Figs. 2A, B). Mean positive response is always lower at high tide than low tide (Fig. 1A), but the curves at each phase of the tide are not significantly different (two way ANOVA). The opposite pattern occurs for negative phototaxis (Fig. 2B) in which at high tide responsiveness is greater at intensities of about 10^{-4} to 10^{-3} W m^{-2}. The curves at each tide are not significantly different but at 8.5×10^{-3} W m^{-2} the overall response levels are different ($P < 0.02$; Z statistic for comparing proportions).

These results suggest that phototaxis varies with phase of the tide. The magni-

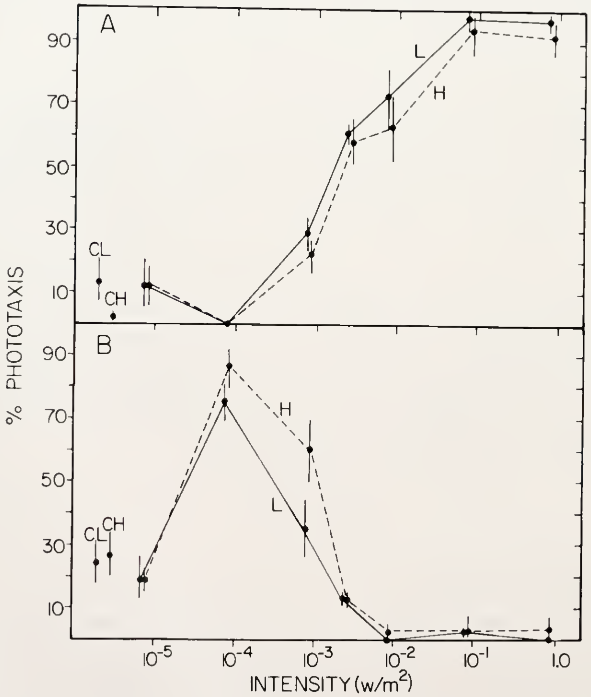

FIGURE 2. Per cent positive (A) and negative (B) phototaxis (ordinate) upon stimulation with a range of light intensities (abscissa). Mean percentages plus standard error are shown. Four trials were conducted with about 30 individuals in each trial. The dashed lines are for high tide (H) and the solid line for low tide (L). Stimulus intensities were the same at both tides, but for clarity, points are slightly offset in plotting. C indicates the control levels at the different tides.

tude of the variation is probably greater than shown because high and low tides were arbitrarily chosen as the time for measurements. Since rhythms in phototaxis are most easily demonstrated by measuring response variation over time upon stimulation with a single intensity, Figures 2A and 2B are useful for indicating the proper intensity range. The values chosen for stimulation are between 6.6×10^{-3} and 1.4×10^{-2} W m^{-2} because these intensities are non-saturating and fall in the area where the sign of phototaxis is changing.

Although Figure 3 shows a rhythmic change in phototaxis, the Fisher periodogram test does not indicate a significant frequency. This is probably due to the rhythm being measured over a relatively short time, showing consistent periodicity early in the records and being less regular in later measurements.

The general pattern is one in which negative phototaxis rises to its greatest level around the middle of the rising tide and declines to a minimum during falling tides. Positive phototaxis has an opposite pattern of greatest responsiveness during falling tides and lowest on rising tides. The rhythm is more pronounced in the second trial (Fig. 3B), probably because a lower stimulus light intensity was used. Clearly, the rhythm is related to natural tidal times, since the timing of the rhythm advances by about the same time (8 hr) as tidal times change from the first to second test dates. Furthermore, the rhythm is endogenous, since it persists under constant conditions for at least 40 hr. The pattern is not as pronounced after this time.

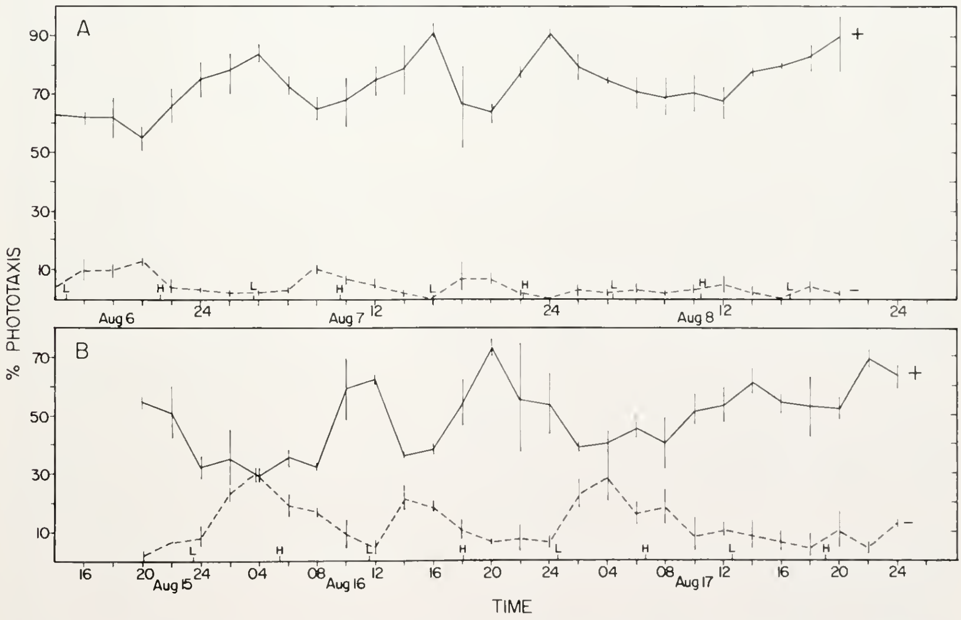

FIGURE 3. Rhythm in positive (solid line) and negative (dashed line) phototaxis (ordinate) over time (abscissa) for larvae collected on August 6, 1979 (A) and August 15, 1979 (B). Mean and standard error are shown at each time. Three trials were conducted with about 30 individuals in each trial. L is time of low tide, and H is time of high tide. The stimulus intensity in A was 6.6×10^{-3} W m^{-2} until 0200 on August 7 when it increased to 1.4×10^{-2} W m^{-2} as the lamp was replaced. In B the stimulus intensity was 7.4×10^{-3} W m^{-2}.

Discussion

Many intertidal organisms have tidal rhythms in behavior, physiology, and reproduction (reviewed by Palmer, 1973). Until the recent demonstration of an endogenous tidal vertical migration of *R. harrisii* larvae (Cronin and Forward, 1979), tidal rhythms in plankton were unreported. The present study presents further evidence of endogenous tidal rhythmicity in larvae of this species. The presence of tidal rhythms in an estuarine plankter is perhaps not surprising, since estuaries frequently have pronounced tidal currents, and the varying behavior may allow the animals to exploit different phases of the tide. Thus it may be constructive to consider the possible functions of the rhythms in swimming speed and phototaxis of *R. harrisii* larvae.

In studies of tidal rhythms, the activity patterns of organisms have perhaps been most often measured (Palmer, 1973). The rhythmic changes in swimming speed (Fig. 1) displayed by *R. harrisii* larvae represent an activity rhythm. The pattern shows highest activity during rising tides, with a maximum usually several hours after low tide, and lowest activity during falling tides, with the minimum occurring several hours after high tide. This pattern could be the basis of the tidal rhythm in vertical migrations observed in the laboratory (Cronin and Forward, 1979).

In the laboratory-observed migrations, the larvae descend to a maximum depth at about the time of low tide and rise to a minimum depth at approximately high tide. The larvae are actually migrating over a short vertical distance of 1 to 1.5 m. The minimum time for the ascent and descent over this distance is 3 hr (Cronin and Forward, 1979). Since the minimum swimming speed observed in the rhythm study (Fig. 1) is about 2.25 mm/sec or 8.1 m/hr, it is obvious that larvae are not swimming directly up or down. It is also doubtful that the larvae are only sinking during the descent, since stage IV zoeae passively sink at a rate of 33 m/hr (15‰ salinity, 23–25° C; Latz and Forward, 1977), which far exceeds the rate of descent.

Alternatively, their depth could change with alterations in activity. If we assume that larvae migrate about some constant depth and that swimming speeds contribute to the direction of migration, then it is reasonable to postulate that at the average swimming speed, the larvae are just maintaining a constant depth in the water. Thus, when the swimming speeds are above the average, the larvae would rise, and, conversely, when swimming speeds are below the average, descent would occur. It is noteworthy that these average swimming speeds occur at times very near the tidal extremes (Fig. 1), and this corresponds closely to the times at which the migrations in constant conditions in the laboratory are at their limits (Cronin and Forward, 1979). Following low tide, the speeds are above average, which leads to a rise. The ascent decreases to zero near the time of high tide, when the average speed is again reached. After this, speeds are below average and the descent to the low tide depth would again occur. Thus the changes in swimming speed can lead to the laboratory-observed tidal vertical migration pattern.

The tidal vertical migration pattern observed in the field shows variable timing (Cronin, 1979). During spring tides when the water column is well mixed, larvae reach a minimum depth about 3 hr before the salinity maximum. On neap tides when more stratified conditions exist, larvae reach minimum depth 4.5 hr after the salinity maximum. Even though the activity rhythm (Fig. 1) was measured at the time of both neap and spring tides, it is very similar on both occasions. Considering the variable timing of the field tidal rhythms (Cronin,

1979) any relation between the activity rhythm in the laboratory and field observations is perhaps fortuitous. Nevertheless, if the activity rhythm is the basis for tidal migration in the field, then a pattern similar to that at spring tide would be predicted.

The activity pattern and associated tidal vertical migration would be adaptive for retention in estuaries. Stratified partially mixed estuaries have a layer of high salinity water with a net landward flow which underlies an upper layer of seaward-flowing lower-salinity water (Pritchard, 1952). If an animal partitions its time between these layers, it is possible to reduce displacement from the parent population. The observed tidal migration would serve this purpose. On rising tides activity would increase, and larvae would move up in the water column and be transported up the estuary. As the tide falls, activity is reduced and larvae descend. Seaward transport would be reduced because the slowest seaward flowing currents occur at greater depths.

Tidal rhythms in phototaxis among animals are not widely studied, and the only reported case is for a sand beach amphipod (Forward, 1980). In *R. harrisii* the rhythm consists of an increase in negative phototaxis to its greatest level around the middle of the rising tide and a decrease to a minimum during falling tide. The opposite pattern is seen for positive phototaxis.

Since phototaxis involves both sensory perception and directional swimming, it is possible that the phototactic rhythm simply results from the activity rhythm. This seems unlikely because the phototactic rhythm consistently continues for only 40 hr while the activity rhythm is still present after 54 hr. Furthermore, if the changes in activity cause alteration in phototaxis, then the overall level of both positive and negative phototaxis should cycle together as responsiveness increases (activity increase) and decreases. This does not occur (Fig. 3).

A more plausible interpretation is that the phototactic pattern is actually changing rhythmically. This is suggested by the data shown in Figure 2. The field captured larvae are light adapted and show the typical phototactic pattern (Forward and Costlow, 1974) of laboratory reared larvae. This consists of a positive response to high light intensities which becomes negative at low intensities. Positive phototaxis is consistently but not significantly lower and negative phototaxis significantly greater at the end of the rising tide (high tide) than at the end of the falling tide (low tide). The difference in phototaxis would be greater if measurement were made at times of greatest negative and positive responsiveness. In the rhythm experiments the stimulus intensity range chosen (10^{-3}–10^{-2} W m^{-2}) evokes both positive and negative phototaxis.

The sign of phototaxis varies rhythmically in that on rising tides negative phototaxis increases and the positive response decreases, with the opposite occurring on falling tides. Thus a discussion of functional significance must consider the change in the phototactic pattern over the tidal cycle.

The phototactic response pattern by *R. harrisii* larvae varies with light and dark adaptation and the light stimulus intensity. Upon light adaptation, the larvae are positive to moderate and high light intensities but become negative to lower intensities. The pattern reverses upon dark adaptation. Forward (1974b) originally hypothesized that the changes in phototaxis upon light and dark adaptation could lead to a diurnal vertical migration pattern involving an ascent at sunrise and descent at sunset. After considering larval photophysiology and rates of light intensity change underwater, Forward and Cronin (1978) concluded that it is very unlikely that the changing phototaxis patterns could result in diurnal vertical migration. Furthermore, Cronin's (1979) field study demonstrated that larvae are generally nearer the surface at night than during the day, which is the reverse of the predicted diurnal vertical migration pattern.

Alternatively, phototaxis may participate in the tidal vertical migration. A generalized pattern based on the activity rhythm (Fig. 1), endogenous vertical movement (Cronin and Forward, 1979) and the field study of migration (Cronin, 1979) indicates that the larvae ascend on rising tides and descend on falling tides. It is questionable that phototaxis contributes to this migration because during the ascent there is an increase in negative and a decrease in positive phototaxis, while the opposite pattern occurs during the descent. Since positive response would lead to an ascent and negative to a descent, this rhythm in phototaxis would actually move the larvae in the opposite directions from those shown during tidal vertical migration. Thus there is no compelling evidence that phototaxis contributes to vertical migration by *R. harrisii* larvae.

Although speculation about the functional significance of diel vertical migration is extensive, perhaps the most common suggestion is predator avoidance. Zooplankton showing nocturnal and twilight migration patterns ascend into suface areas at times when illumination is low (night) and descend from these areas during the day, thereby avoiding visually oriented predators. In field studies, *R. harrisii* larvae primarily show a tidal migration pattern which has a diel component of shallower distributions at night than during the day (Cronin, 1979). This diel migration may also reflect predator avoidance. Nevertheless, because larvae migrate according to tides, they must move into shallow depths at some time during the day. In this area they would encounter visually oriented predators as well as those which swim slowly near the surface, such as ctenophores (See Forward, 1976, for a detailed discussion).

The most unusual aspect of larval phototaxis is the negative response to low light levels when light adapted. A series of studies (Forward, 1974b, 1976) supported the hypothesis that this negative phototaxis is part of a shadow response used for avoidance of predators which do not actively pursue their prey and occur close to the surface, such as ctenophores and coelenterate medusae.

The tidal rhythm in phototaxis shows an increase in negative responsiveness on rising tides. This is also the time the larvae ascend in the water column into better-illuminated areas. Thus, it is plausible to hypothesize that the functional significance of the phototactic rhythm is predator avoidance, in which the negative phototactic part of the shadow response is increased at times when the larvae are higher in the water column and thereby more likely to encounter ctenophores and coelenterate predators.

The present study presents further evidence of tidal rhythms in estuarine plankton. The occurrence of similar rhythms in other estuarine plankton and the aspects of the estuarine tidal cycle that actually entrain the rhythms remain to be determined.

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SUMMARY

1. Tidal rhythms in swimming speed and phototaxis were measured in stage III zoeae of the crab *Rhithropanopeus harrisii.* Larvae were captured in the Newport River estuary, North Carolina, and maintained under constant conditions in the laboratory.

2. Swimming speed increases during rising tide with a maximum several hours after low tide, and decreases to a minimum about 2 hr after high tide. It is argued that this pattern is the basis of the endogenous tidal vertical migration, in which the larvae ascend on rising tides and descend on falling tides.

3. The tidal rhythm in phototaxis consists of an increase in negative responsiveness which is strongest around the middle of the rising tide, and a decline to a minimum during falling tides. Positive phototaxis shows the opposite pattern. There is no convincing evidence that phototaxis participates in vertical migration. It is hypothesized that the phototactic pattern represents a tidal rhythm in a shadow response used for predator avoidance.

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"CONTACT REACTIONS" BETWEEN XENOGENEIC OR ALLOGENEIC COELOMIC CELLS OF SOLITARY ASCIDIANS

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The ability to distinguish self and nonself components seems to be widespread throughout the animal kingdom (Hildemann and Reddy, 1973; Cooper, 1976). The vertebrates, from the cyclostomes onward, are capable of mounting specific humoral as well as cell-mediated immune responses in defence against invading nonself components. In invertebrates, however, it is only in advanced organisms that recognition of foreignness is followed by selective destruction or elimination of such material, and even then this is accomplished only by means of cell-mediated mechanisms. It is as yet unclear whether invertebrates possess an ability for "adaptive immunity" as defined by the presence of specific anamnesis, even though primitive forms of "memory" have been demonstrated in certain annelid worms (Cooper, 1969). It would seem likely, therefore, that a sharp gap exists between vertebrates and invertebrates in the evolutionary stages attained by their defence mechanisms. However, evolution of the mechanisms by which self and nonself cell-surface components are recognized in the animals is itself of considerable interest, quite apart from the origin of active rejection mechanisms subsequent to nonself recognition. The importance of such self–nonself recognition beyond its role in self-defence is underlined by the fact that the genes of the major histocompatibility complexes of higher vertebrates are known to control physiological interactions between syngeneic cells (Katz, 1977).

Ascidians, a group of urochordates, occupy a unique position in the metazoan phylogeny, as these animals are often regarded as related to the immediate forerunners of the vertebrates. Studies of self–nonself recognition in ascidians are therefore particularly relevant, since such studies might reveal crucial evolutionary steps towards the sophisticated cell-interaction systems of vertebrates. From this point of view, extensive studies of the colonial ascidians have been particularly rewarding. Using *Botryllus primigenus,* Oka and his colleagues have shown that colonies which share an allele of the single "histocompatibility" locus fuse with each other, whereas colonies lacking a shared allele exhibit mutual non-fusion reactions (Oka and Watanabe, 1957; Oka, 1970; Tanaka and Watanabe, 1973). The genetic control of allogeneic reactivity in this system thus follows a rule which clearly differs from the familiar rules of transplantation genetics established for the vertebrates. Nevertheless, these animals must possess genetically specific recognitive ability for self components, coded for or controlled by the "histocompatibility" locus. Moreover, a strict correlation was found between alloreactivity and the ability of gametes from two colonies to fertilize each other, raising an intriguing possibility that the "histocompatibility" locus in the ascidians may control cell-to-cell interactions in general (Oka and Watanabe, 1957; Oka, 1970).

Solitary or simple ascidians have so far received much less attention than the colonial or compound ascidians. In a previous paper, we reported the nature of naturally occurring hemagglutinins in the coelomic fluid of such ascidians. The substance(s) appeared to be a mucopolysaccharide unrelated to immunoglobulins (Fuke and Sugai, 1972). I now report studies on a nonphagocytic cellular reaction of the solitary ascidians against xenogeneic and allogeneic cells. When isolated coelomic cells from two species of solitary ascidians were brought into contact *in vitro*, both cells reacted against each other, resulting in reciprocal lysis of both cells. Moreover, coelomic cells of one of such species, *Halocynthia roretzi* (Drasche), were studied for their allogeneic reactivity and were found to exhibit the same type of cellular reactions in some but not all combinations of different individuals. This cellular reaction, henceforth called "contact reaction," will be described in detail below. The reaction can be regarded as a cellular counterpart of nonfusion reactions in the colonial ascidians.

MATERIALS AND METHODS

The following six species of solitary ascidians were employed: *Halocynthia roretzi* (Drasche), *H. aurantium* (Pallas), *Pyura mirabilis* (Drasche), *Styela clava* (Herdman), *Ciona intestinalis* (Linnaeus), and *C. robusta* (Hoshino and Tokioka). *H. aurantium* specimens were obtained at Otaru, Hokkaido; the rest were collected in Mutsu Bay, Aomori. All experiments were carried out at the aquarium facilities of the Marine Biological Station, Asamushi, Aomori. Three variants of *H. roretzi*, designated as Types A through C, have been described in detail (Numakunai and Hoshino, 1973).

Coelomic cells of *H. roretzi*, *H. aurantium*, and *P. mirabilis* were collected from the mantle-test interspace by withdrawing body fluid into hypodermic syringes containing a five- to ten-fold excess of sea water. Those from *S. clava* and the two species of *Ciona* were obtained through incisions made in the test and the mantle. Up to 5×10^6 coelomic cells of each animal to be tested were cultured in glass chambers measuring $15 \times 25 \times 1$ mm deep. Unless otherwise stated, xenogeneic or allogeneic reactions were observed as follows: Initially, the coelomic cells from one animal were cultured for 5 min. The culture fluid was discarded and the cells which adhered to glass surfaces were washed several times with fresh sea water before a suspension of cells from a second animal was introduced into the chamber. The chamber was placed under a phase-contrast microscope and the reactions were observed at a magnification of 1000 ×. Normally, cells from different species were readily distinguished by morphological criteria (Fuke, 1979). Cells from the two *Ciona* species, however, were morphologically alike and had to be distinguished from each other by following, under an inverted microscope, the settlement of the cells from the second animal on the adhered cells from the first animal. In experiments involving cells from conspecific variants or individuals, vital staining of cells from one animal served to identify the donor of each cell. The vital stain employed was either Toluidin Blue or Nile Blue, both at 0.001% concentration. The viability and reactivity of stained cells were indistinguishable from those of unstained cells.

Since sperm of one individual could be freed of autologous eggs, but not vice versa, fertilization experiments were carried out by mixing separated spermatozoa from one animal with an egg-sperm mixture from another animal.

RESULTS

The phenomenon of contact reaction and the cell types involved

When coelomic cells of one animal were brought into contact *in vitro* with such cells from a different animal (except for certain allogeneic combinations; see below), a series of cellular events, here termed "contact reaction," ensued. The reaction can be followed by time-lapse photographs: In the first example, a reaction between xenogeneic cells was recorded (Fig. 1). Coelomic cells of *H. roretzi* were cultured for 5 min and washed once with sea water. Cells of *P. mirabilis* were then introduced to the culture chamber. Shortly after the two xenogeneic vesicular cells came into contact (Fig. 1a), the *H. roretzi* cell started moving around the *P. mirabilis* cell, which remained stationary (Figs. 1b and 1c). The former then ceased movement and appeared to press itself tightly to the latter (Fig. 1d). Within seconds, the *H. roretzi* cell was lysed (Fig. 1e), followed immediately by lysis of the *P. mirabilis* cell (Fig. 1f). In this particular pair of cells, the *H. roretzi* cell appeared to initiate the whole process of the contact reaction by assuming the role of an "attacker" cell. However, in many other similar pairs of cells in the same culture, a *P. mirabilis* cell acted as an apparent "attacker." Not infrequently, moreover, two cells in a pair took turns moving around each other, indicating that the two cells in a reacting bicellular conjugate are "attackers" and "targets" at the same time. It was unpredictable which of the two cells in contact would be lysed first, even when the apparent "attacker" could be discerned from the "target." In any case, lysis of one cell was always followed immediately by that of the other cell. In this reaction, therefore, distinction between "attacking" cells and "target" cells is superfluous because of their complete reciprocity. This sharply contrasts the ascidian contact reaction from cell-mediated cytotoxicity in higher animals. Moreover, pairs of cells sometimes proceeded to the "tight adherence" stage without undergoing the initial "maneuver" of one of the cells around the other, suggesting that such preparatory movement is not a prerequisite for subsequent reactions.

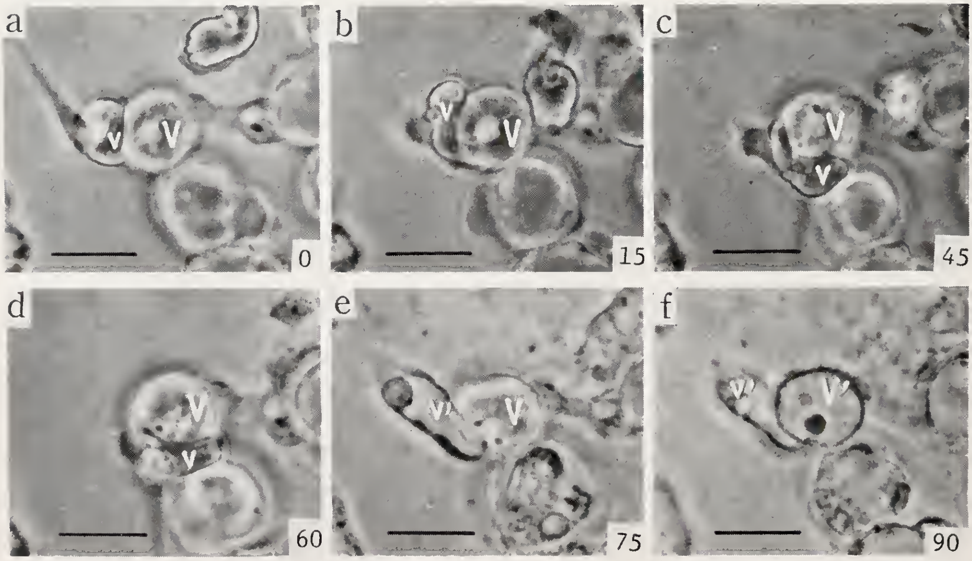

FIGURE 1. Contact reaction between vesicular cells of *P. mirabilis* (V) and *H. roretzi* (v). Upon contact (a), the v cell moves around the V cell (b, c). The movement stops and the v cell presses itself to the V cell (d). Th v cell is then lysed (v′, in e), followed promptly by the lysis of the V cell (V′, in f). Scale bar = 10 μ. The time required for the reaction in seconds is shown at the lower right corner of each picture.

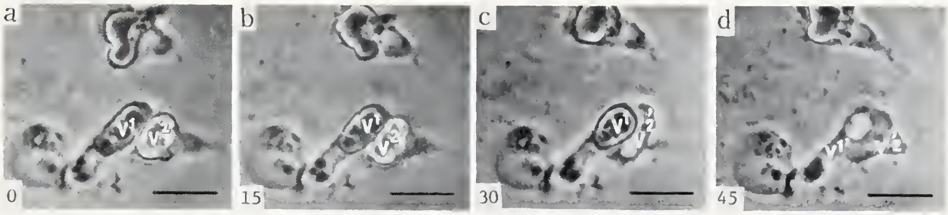

FIGURE 2. Contact reaction between vesicular cells (v^1 and v^2) from two individuals of Type C *H. roretzi.* One cell, v^1 was vitally stained with Nile Blue and appears darker than the second, unstained, v^2 cell. Following contact (a), both cells remain stationary (b), followed by lysis of v^2 ($v^{2\prime}$, in c) and then v^1 cell ($v^{1\prime}$ in d). Scale bar = 10 μ. Time elapsed after the cell contact is indicated in seconds at the lower left corner of each frame.

Xenogeneic contact reactions were observed in congeneric species pairs as well as between species of different genera, for example, *H. roretzi* vs. *H. aurantium, H. roretzi* vs. *P. mirabilis, H. roretzi* vs. *S. clava, P. mirabilis* vs. *S. clava,* and *C. intestinalis* vs. *C. robusta.* The strength of contact reactions in various species pairs, judged by the frequency of reactions and the average time required from cell contact to lysis, was roughly the same except possibly for reactions involving the two *Ciona* species, whose reaction seemed somewhat weaker than that between other xenogeneic combinations examined.

Three variants of *H. roretzi*, designated as Types A, B, and C, have been described by Numakunai and Hoshino (1973). They differ in peak daily spawning hours, reproductive season, and preferred habitat, as well as in external morphology. Cells from different variants, when tested by mixing *in vitro,* exhibited vigorous contact reactions in all combinations of the three types. Although these three variants are taxonomically considered conspecific, genetic differences between any two types are probably intermediate between xenogeneic and allogeneic. It was therefore of interest to test whether cells from different individuals within the same variant could react with each other. In such experiments, contact reactions were observed in most but not all pairs, as discussed below. When an allogeneic contact reaction did occur, however, it was in all respects indistinguishable from xenogeneic reactions. Figure 2 shows an example of allogeneic contact reaction between vesicular cells from two Type C *H. roretzi* individuals. As in xenogenic reactions, the cells, upon establishing contact, adhered tightly to each other (Figs. 2a and 2b). This was followed by lysis of one of the cells (Fig. 2c) and then of the other (Fig. 2d).

The exact nature of "lysis" in these reactions and the fate of "lysed" cells are unclear. Since most of the coelomic cell types involved in this reaction (see below) contain vesicles of various sizes, the "lytic" process is readily detected as discharge of such vesicles from the cells undergoing contact reactions. The vesicles at the same time disintegrate and release their contents into the surrounding medium. A "lysed" cell thus shows vacuoles, and remains totally immobilized for at least several hours while still in tight contact with the other, also "lysed," cell. Following such a motionless period, at least some of the "lysed" cells extended their pseudopodia and apparently loosened themselves from the partners, suggesting that the contact reaction might not necessarily entail cell death.

Contact reactions were observed between cells of the same morphological type as well as between cells of different types. Those which participate in contact reactions are vesicular cells, large granular amoeboid cells, small granular amoe-

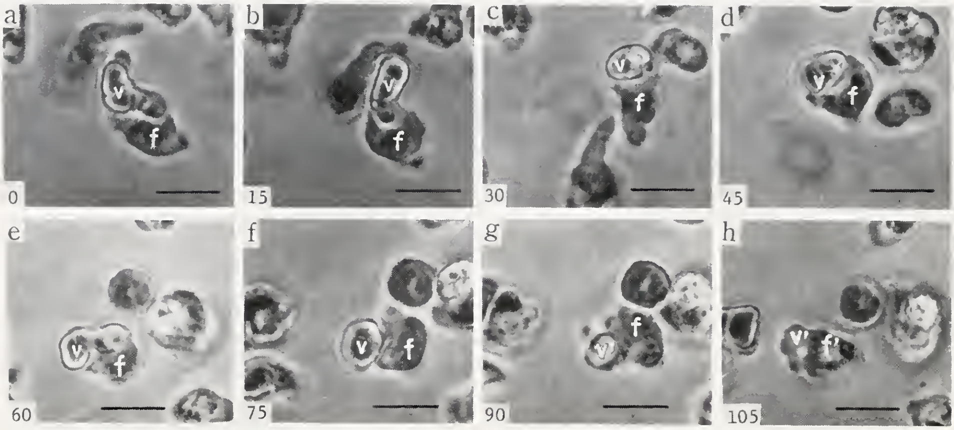

FIGURE 3. Contact reaction between a fine-granular amoeboid cell (f) and a vesicular cell (v) from two Type C *H. roretzi* donors. The f cell has been stained with Nile Blue and is shown darker than the unstained v cell. The f cell moves around the v cell (a–f), until the latter discharges vesicles (v′, in g). The f cell itself is also lysed soon afterwards (f′, in h). The scale bar indicates 10 μ. The time after initiation at which each picture was taken is shown in seconds in lower left corners.

boid cells, minute granular cells, fine granular amoeboid cells, large basophilic cells, and the so-called vacuolated cells that differ from "lysed" cells of various types (for nomenclature and identification of various cell types, see Fuke, 1979). Whether other cell types, such as brown cells, orange cells, and "lymphocytes," also engage in contact reactions has not been determined because of their low frequencies among coelomic cells. An allogeneic contact reaction between a fine-granular amoeboid cell and a vesicular cell is recorded in Figure 3. The former cell initially moved around the latter and the lysis of the latter cell can be seen by its vesicular discharge (Fig. 3d).

Requirement for direct cell contact

Spontaneous lysis of coelomic cells was never observed in cultures of cells from a single animal, regardless of whether or not the cells were in contact with another autologous cell. Hence, autologous contact reactions do not occur. In mixed cultures of nonautologous cells, except for certain combinations of allogeneic cells, cytolysis was apparently triggered only when two heterologous cells came into direct contact. However, under the experimental conditions employed, most of the mixed cultures contained some coelomic fluid carried over from one or the other cell donor. It seemed conceivable, therefore, that the presence of heterologous body fluid made cells more susceptible to spontanous lysis and that, if so, at least some of the cells might be lysed without direct cell contact. Once such spontaneous cytolysis did occur, it could possibly lead to nonspecific lysis of proximate cells (see below). Effect of heterologous coelomic fluid on spontaneous lysis was therefore tested. Additionally, culture media in which vigorous contact reactions had previously taken place were also tested to see if incidence of spontaneous lysis could be increased when fresh cells were deliberately exposed to such media.

Cell-free coelomic fluid from *H. roretzi* or *P. mirabilis* was obtained by centrifugation. Cells from one or the other species were cultured in glass chambers, washed several times with sea water and then cultured with coelomic fluid from either species. Regardless of the source of fluid, cells of these two species were perfectly normal during several hours' observation and showed no indication of spontaneous lysis.

Coelomic cells from *H. roretzi* and *P. mirabilis* were cocultured for 30 min, a period long enough to ensure completion of contact reactions, and the culture medium (*i.e.,* mixed coelomic fluid) was collected. Fresh *H. roretzi* or *P. mirabilis* cells then were cultured separately in the medium containing material released from lysed cells. Cells from either animal in such cultures behaved exactly like the cells in control cultures for which fresh autologous coelomic fluid was used. Similar experiments also were carried out with cells from two alloreactive individuals using media in which allogeneic contact reactions took place, and the results were the same.

These experiments confirm the absence of spontaneous lysis and autologous contact reactions in xenogeneic or allogeneic mixed cultures. In addition, they strongly suggested that a contact reaction is a specific reaction triggered by direct cell contact between xenogeneic or allogeneic cells. However, in contrast to the requirement of direct cell contact in triggering contact reactions, effector mechanisms of these reactions may not necessarily require cell contact. When contact reactions between vesicular cells of *H. roretzi* and *P. mirabilis* took place, some of the nearby *P. mirabilis* vesicular cells were found to be lysed without prior contact with *H. roretzi* cells. In fact, lysis of one cell appeared to trigger a chain reaction among nearby vesicular cells. Since supernatant from cultures in which contact reactions had taken place did not have such an effect, the factor(s) which caused this chain reaction either was labile or was effective only over a short range. Vesicular cells of *P. mirabilis* contain particularly large vesicles and they tended to be more sensitive to routine manipulations than other cells, perhaps accounting for the observation of such spontaneous lysis only in this cell type. Whether or not this short-range reaction in the absence of cell contact is analogous to the lysis initiated by cell contact remains to be elucidated. It seems possible, however, that lysis of the second cell in a cell pair undergoing contact reaction is a direct consequence of lysis of the first cell, the two cells being as close to each other as they can. This observation indicates that cytolysis can be mediated by diffusible material, probably derived from vesicles. Nevertheless, it should be emphasized that lysis of the first cell in a bicellular conjugate is in all likelihood effected by direct cellular interactions.

Finally, despite the lack of autologous contact reaction between healthy cells, it is conceivable that degenerated cells are eliminated by autologous cells via a similar reaction. To test this, fresh *H. roretzi* cells were mixed with autologous cells that had been cultured for up to 12 hr. Even under such circumstances, no autologous reactions ensued, presumably because such reactions do not play a role in removing aged cells, or the culture period was too short to produce cells sufficiently degenerated to elicit autologous reactions.

Contact reaction in vivo

The above observations are concerned only with contact reactions in an artificial, *in vitro,* system. An obvious question is whether the same type of reaction

TABLE I

Reciprocal contact reactivities among 16 Type A individuals of H. roretzi. *Plus (+) and minus (−) denote presence and absence of contact reactions, respectively.*

	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	No. 9	No. 10	No. 11	No. 12	No. 13	No. 14	No. 15	No. 16
No. 1	−	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+
No. 2	+	−	+	+	+	+	+	+	+	+	+	−	+	+	+	+
No. 3	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+
No. 4	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+
No. 5	+	+	+	+	−	+	−	+	+	+	+	+	+	+	−	+
No. 6	+	+	+	+	+	−	+	+	+	+	+	−	+	+	+	+
No. 7	+	+	+	+	−	+	−	+	+	+	+	−	+	+	−	+
No. 8	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+
No. 9	+	+	+	+	+	+	+	+	−	+	+	+	+	+	−	+
No. 10	+	+	+	+	+	+	+	+	+	−	−	+	−	−	+	+
No. 11	+	+	+	+	+	+	+	+	+	−	−	+	+	−	+	+
No. 12	+	−	+	+	+	−	−	+	+	+	+	−	+	+	+	+
No. 13	−	+	+	+	+	+	+	+	+	−	+	+	−	−	+	−
No. 14	+	+	+	+	+	+	+	+	+	−	−	+	−	−	+	+
No. 15	+	+	+	+	−	+	−	+	−	+	+	+	+	+	−	+
No. 16	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	−

can be demonstrated in intact animals. To answer this question, coelomic cells of *P. mirabilis* were marked with a vital stain and their suspensions in a volume of 0.4 ml were injected between mantle and test inside the papillae of *H. roretzi.* Twenty min later, coelomic fluid from the site was withdrawn with a syringe and the cells were examined microscopically. None of the donor type, *i.e.,* stained, cells were single, all being surrounded by one or more unstained, host-derived cells. About $\frac{2}{3}$ of the donor cells had vacuoles, indicating vesicle discharge following contact reactions. The rest of the cells had normal vesicles, *i.e.,* had not been lysed. Therefore, the timing and features of *in vivo* reactions were quite similar to the observations made *in vitro,* strongly indicating that a contact reaction can be induced *in vivo* as well as *in vitro.*

Pattern of alloreactivity in H. roretzi

As already pointed out, cells from different individuals of the same variant of *H. roretzi* exhibited contact reaction in most but not all combinations of individuals. In a preliminary experiment, for instance, cells from one Type C individual were tested against those from six other Type C animals. One of the six failed to react with the first individual. In another experiment with the Type C animals, cells from one individual did not react with those from one out of nine individuals tested. Thus, cells from at least some individuals behaved as if they were autologous. However, two individuals which do not react wth each other are not necessarily identical, since they might react differently with a third individual. It was, therefore, of considerable importance to analyse patterns of reciprocal reactivity by using a large panel of individuals.

Sixteen Type A individuals were collected from an area of approximately 10 m^2 near Futago-jima Island in Mutsu Bay, and were subjected to mixed culture experiments in all possible combinations. The results of this experiment, involving a total of 120 combinations, are shown in Table I. Three individuals

(Nos. 3, 4, and 8) reacted with all others, whereas one animal (No. 13) failed to react with as many as four other individuals. Thus, the frequency of non-reactive allogeneic combinations for each individual ranged from 0/15 to 4/15. All except two of the 16 animals were different in their alloreactivity patterns. For instance, animals No. 5 and 7 did not react with each other and showed parallel reactivity towards Nos. 9 and 15, yet differed in reactivity against animal No. 12. Two exceptions were Nos. 10 and 14. They did not react with each other and were identical in being reactive with all others except for Nos. 11 and 13. Thus, these two behaved as if they were "syngeneic" in histocompatibility as far as this test was concerned. Obviously, however, these two might have reacted differently had another individual been added to the test panel.

Lack of correlation between alloreactivity and fertilization

Induction of a contact reaction requires direct contact between cells, and hence it involves mutual recognition of cell surface components. A vital, naturally occurring process which depends on such cellular recognition is fertilization. In the solitary ascidians studied here, each individual simultaneously releases sperm and eggs through the atrial aperture. However, self-fertilization, *i.e.,* fertilization between autologous spermatozoa and eggs, is known to occur, usually at a low rate (1–5%). In contrast, the frequency of fertilization between gametes from different individuals is as high as 80 to 90%. This well-known self sterility raises an interesting question, namely whether there is a correlation between alloreactivity and the frequency of fertilization. In a colonial ascidian *Botryllus primigenus,* Oka and Watanabe (1967) found a strict correlation between fertilization and "non-fusion" reaction, an expression of alloreactivity which in many respects resembles contact reactions of solitary ascidians described in the present study. In the colonial ascidians, fertilization occurred only between alloreactive colonies, suggesting that cell surface components involved in fertilization and alloreactivity are under the same genetic control.

To test if the rules observed in the colonial ascidians also apply to solitary ascidians, the frequency of fertilization between pairs which are either positive or negative in contact reactivity was examined. In the first experiment, the frequency of fertilization between two individuals that showed contact reaction (Nos. 8 and 9 in Table I) was found to be about 94% and the majority of fertilized eggs developed into tadpoles (Experiment 1, Table II). In subsequent experiments, however, similar high frequencies of fertilization were obtained even between individuals which did not exhibit contact reactions, for instance between animals Nos. 10 and 11, or between Nos. 13 and 16, irrespective of which one of the pair provided the egg or the sperm (Experiments 2 and 3, Table II). These results therefore indicate that the alloreactivity in terms of contact reactions is irrelevant for fertilization.

DISCUSSION

Ascidians are capable of recognizing foreign material and of eliminating it from their bodies. In a solitary ascidian, *Molgula manhattensis,* hemocytes either encapsulate or phagocytize such material for removal (Anderson, 1971). Coelomic cells of solitary ascidians phagocytize and eliminate sea urchin spermatozoa and fixed mammalian erythrocytes (Fuke, 1979). Observations reported in the pres-

TABLE II

Lack of correlation between contact reactivity and fertilization in H. roretzi *Type A individuals. The animals tested were some of those employed in the experiments shown in Table I, and are assigned the same numbers.*

	Contact reaction of coelomic cells	Developmental stage 48 hr after mixing (% of total scored)		
		Uncleaved egg	Cleavage stage, blastula, gastrula, or neurula	Tadpole
Exp. 1				
No. 8 (egg + sperm)		84	14	2
No. 9 (egg + sperm)		85	12	3
No. 8 + No. 9 (both are egg + sperm)	+	6	13	81
Exp. 2				
No. 10 (egg + sperm)		96	0	4
No. 11 (egg + sperm)		89	3	8
No. 10 (egg) + No. 11 (sperm)	−	8	5	87
No. 11 (egg) + No. 10 (sperm)	−	23	0	77
Exp. 3				
No. 13 (egg + sperm)		81	17	2
No. 14 (egg + sperm)		86	12	2
No. 16 (egg + sperm)		89	7	4
No. 13 (egg) + No. 16 (sperm)	−	18	15	67
No. 16 (egg) + No. 13 (sperm)	−	11	29	60
No. 14 (egg) + No. 16 (sperm)	+	2	8	90
No. 16 (egg) + No. 14 (sperm)	+	2	12	86
No. 13 (egg) + No. 14 (sperm)	−	8	12	80
No. 14 (egg) + No. 13 (sperm)	−	9	21	70

ent paper reveal another function of coelomic cells from the solitary varieties of ascidians, namely the recognition and destruction of xenogeneic and allogeneic cells via "contact reactions." The reaction is triggered when coelomic cells from two different animals come into contact, though there are exceptional situations in which two allogeneic cells do not react, as discussed further below. The reaction results in almost simultaneous lysis of both cells. Thus, the reaction is bidirectional and ends in a "forced suicide" for both parties. Unlike conventional cell-mediated cytotoxicity of higher vertebrates, an effector-target cell relationship does not seem to hold between two cells in a contact reaction. At least in some cases, diffusible material released by lysis of the first cell may lead to lysis of the second cell and of a nearby "bystander." Nevertheless, the inference is that lysis of the first cell is directly effected by interactions at the membrane level. The absence of autologus contact reactions under a variety of conditions implies involvement of a self-nonself recognition system in this reaction. The effector mechanism, as noted above, may include both cellular and diffusible mediators. A number of morphologically distinct cell types are capable of performing this reaction. However, nothing is known about interrelationships among these cell types, or how they are related to immunocompetent cells of higher vertebrates. Contact reactions were initially found in mixed cultures of xenogeneic or allogeneic coelomic cells and in fact most of the experiments reported here were carried out *in vitro.* However, similar reactions presumably take place in intact animals if foreign cells have been accidentally introduced into their bodies; inoculation of coelomic cells from one animal into the mantle-test interspace of another animal resulted in lysis of donor cells following formation of cell aggregates between the donor- and host-derived cells.

As already noted, this cellular reaction occurs between allogeneic as well as xenogeneic cells. The sequence of events, the consequence, and the type of cells involved were the same whether the reaction took place in xenogeneic or allogeneic combinations. Allogeneic contact reactions, however, could be observed in most but not all combinations of individuals. A checkerboard-type experiment of 16 *H. roretzi* Type A individuals afforded 120 different reciprocal combinations. Of these, 14 combinations or roughly 12% did not evoke contact reactions (Table I). When alloreactivity of each of the 16 animals was compared, only two individuals had identical patterns and each of the rest of the panel displayed a unique pattern. In addition, three individuals out of 16 (19%) had alloreactivity with all the remaining individuals. These results cannot be explained by applying the familiar rules of transplantation genetics established for higher vertebrates. Absence of alloreactivity between two individuals of the mouse, for instance, indicates lack of disparity in their histocompatibility antigens. Accordingly, these two mice will react exactly the same way against histocompatibility antigens of a third mouse. In the ascidians, however, two histocompatible individuals more often than not differ in their reactivity with a third individual of the same species. For example, animals Nos. 2 and 12 do not react with each other, yet animal No. 6 reacted with No. 2 and not with No. 12, indicating that the former two individuals could not be identical (Table I). Obviously, therefore, alloreactivity in ascidians is governed by a rule different from the one applicable to higher vertebrates. The exact nature of genetic control for alloreactivity of *H. roretzi* must be elucidated employing larger panels of randomly collected individuals.

Most ascidians are hermaphrodites with varying degrees of self-sterility: Normally, fertilization occurs only between gametes from different individuals. In the solitary ascidian, *Ciona intestinalis,* this self-sterility is due to a blockade at the egg membrane (chorion) which prevents penetration of autologous spermatozoa into the egg (Morgan, 1923, 1943). Removal of the membrane by mechanical means makes such eggs fertilizable not only by autologous spermatozoa (Morgan, 1923) but even by spermatozoa from species of other genera, leading in the latter case to the development of interspecific hybrids at high frequencies (Minganti, 1948). It is in this regard that the positive correlation between alloreactivity and fertilizability in the colonial ascidian *B. primigenus* is particularly intriguing, since it raises the possibility that a single histocompatibility locus may control not only the alloreactivity but also other cell-to-cell interactions (Oka and Watanabe, 1957, 1967; Oka, 1970). Rather unexpectedly, however, the results of similar experiments with the solitary *H. roretzi* showed no apparent relationship between allogeneic reactivity and fertilization (Table II). This was not due to indiscriminate fertilization, because self-fertilization frequencies were consistently low in every individual tested. Whatever the exact differences in the mechanism of genetic control of fertilization may be (*e.g.*, germ-line versus somatic origin of egg membrane, surface recognition mechanism, etc.), the difference in the restriction for fertilization strongly suggests that the genetic structures of the populations of these two species are dissimilar.

Studies of self-nonself recognition in invertebrates by means of xenograft or allograft rejections have been hampered by the long observation periods normally required for end-point determination. In contrast, xenogeneic or allogeneic contact reactions are complete within a few minutes. Moreover, there is no need to sacrifice the donors to remove coelomic cells. Although it remains to be seen if analogous reactions obtain in animal groups further down the phylogenetic tree, the *in vitro* contact reactions should prove to be an excellent model for genetic and biological studies of primitive self-nonself recognition at the cellular level.

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SUMMARY

Coelomic cells from solitary ascidians exhibit a nonphagocytic cellular reaction when they are mixed *in vitro* with such cells from different species or another individual of the same species. The reaction is triggered by direct contact between reactive cells and hence was denoted "contact reaction." The contact reaction is reciprocal and results in mutual cell deaths or a state of prolonged incapacitation of both cells involved. Coelomic cells of several morphological types are capable of carrying out the reaction. Similar reactions were also observed *in vivo* when foreign cells were deliberately introduced into intact animals.

Individuals from a single variant type of *Halocynthia roretzi* (Drasche) are mutually reactive when their cells are brought into contact, but not in every combination of individuals. The patterns of positive and negative contact reactivity among such allogeneic individuals do not follow conventional rules of transplantation genetics established for the vertebrates. These reactions seem to represent a cellular counterpart of non-fusion reactions in the colonial ascidians. However, unlike the colonial ascidians, in which fertilization between gametes from two individual colonies correlates with positive alloreactivity between them, that of solitary *H. roretzi* apparently takes place between any two individuals regardless of their mutual alloreactivity.

The *in vitro* contact reaction is complete within a few minutes. Since coelomic cells can be tapped without sacrificing the donor animals, the *in vitro* contact reaction promises to be an excellent model for further studies of primitive self-nonself recognition at the cellular level.

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THE EFFECT OF SUSPENSION DENSITY ON THE RETENTION OF 5 μM DIATOMS BY THE *MYTILUS EDULIS* GILL[1]

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Wallengren (1905) observed that the latero-frontal cirri, the compound filtering cilia on the gill surface of certain bivalve molluscs (the macrociliobranchia of Atkins, 1938; revised by Owen, 1978), have a series of branches. Carter (1924) and Dral (1967) stated that these branches were produced by degradation and that a healthy cirrus is an unbranched structure that traps particles by adhesion. Recent electron microscope studies (Moore, 1971; Owen, 1974) have verified Wallengren's observation and shown that in *Mytilus edulis*, the distance between branches of the cirrus is 0.6 μm, seemingly with little variation. For the simple case of spherical particles, a perfect retention of 0.6-μm diameter is thus predicted given that a) the branches of cirri act like sieves, b) escape of particles as they impinge on the filter is negligible and c) alternate cirri of the same filament beat synchronously (*i.e.*, regular coordination). Since branches of alternate cirri overlap, condition c), which Owen (1974) considered as "normal," means that the ostial opening is covered by the cirral meshwork at all times. (Only spherical particles are considered in this paper, so that the retention properties of the filter can be characterized by a minimum mesh dimension.)

However, Dral (1967) observed young semitransparent specimens of *M. edulis* in dense algal suspensions and concluded that certain variations in cirral beat could alter the gill's retention efficiency, E%, for particles as large as 20 μm. Although Dral based his estimates on the incorrect assumption that the cirri are unbranched, his observations concerning irregular coordination and halt positions of cirri could still predict a reduced retention of relatively large particles. Since the ostial channel (the gap between adjacent filaments) can be 30- to 40-μm wide, such predictions are theoretically possible. Jorgensen (1975) has indicated that serotonin, by direct action on the positioning of the latero-frontal cirri, may decrease E% for 5-μm yeast cells. Furthermore, Owen (1974) observed that up to two pairs of adjacent cirri on the same filament could beat synchronously and that the activity of the cirri showed "considerable variation." Davids (1964) estimated E% values as low as 20 and 25% for the unicellular algae *Chlorella* sp. and *Isochrysis galbana* (both approximately 5.0-μm diameter). Furthermore, he observed that E% could vary inversely with food concentration.

The present study investigates E% in *Mytilus edulis* at various concentrations of *Thalassiosira pseudonana* (a concentric diatom whose volume, determined by the coulter-counter, equals a sphere of 5-μm diameter) in an attempt to reconcile the above discrepancies between the empirical data and the predictions. In particular, the purposes of the report are a) to re-examine the observations of Davids (1964) and b) to determine if the mechanisms observed by Dral (1967) have any measurable effect on E% for 4.4- to 6.4-μm particles. The discussion will

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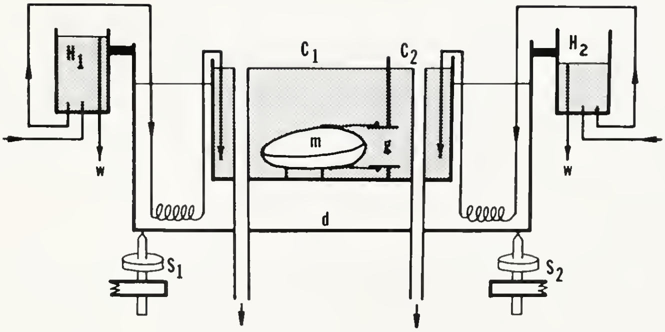

FIGURE 1. Diagrammatic representation of the apparatus used for estimation of gill retention efficiency and pumping rate. Arrows indicate the direction of water flow. W signifies flow to waste from the constant heads. For explanation of other lettering, see text.

include how cirral morphology and function can account for some published observations of E% for various particle sizes.

MATERIALS AND METHODS

Exhaled water was separated from specimens of the mussel, *M. edulis,* by attaching lightweight rubber sleeves to the valves around the exhalant aperture regions. A fuller account of the following description is given by Hildreth (1976).

The apparatus is shown in Figure 1. The water bath, d, is supported on three screws (only two, S_1 and S_2 are shown) which enable the water levels in chambers C_1 and C_2 to be altered with respect to one another. To ensure that the hydrostatic pressure difference between C_1 and C_2 is zero, the following pre-adjustments are made: 1) with connecting pipe g closed, the overflow rates from C_1 and C_2 (supplied by constant heads H_1 and H_2, respectively) are measured; 2) with g open, screws S_1 and S_2 are adjusted so that the overflow rates from C_1 and C_2 are equal to their respective input rates from H_1 and H_2. (H_1 and H_2 supply rates to C_1 and C_2 are 250 and 25 ml/min, respectively.)

The mussel's rubber sleeve is then connected to pipe g so that the mussel, m, inhales from C_1 and exhales into C_2. The mussel's pumping rate, r_b, is estimated as the volume overflow rate from C_2 minus the constant head input from H_2. The overflow rate from C_2 is recorded automatically. Diatom culture is fed into the water supply of H_1.

E% was estimated for 4.4- to 6.4-μm-diameter particles by taking discrete samples simultaneously from the overflows of C_1, C_2, and H_2. Samples were immediately analyzed for particle concentrations (by numbers/ml) using a model Z_B coulter-counter with 140-μm tube. Samples were counted in quadruplicate with a 0.5 ml manometer. The particle concentration of the mussel's exhaled water $[C_2]'$ was calculated as:

$$[C_2]' = ([C_2] - r_a[C_3]/R)R/r_b \tag{1}$$

where $[C_2]$ is the particle concentration of water leaving C_2, $[C_3]$ is the concentration leaving H_2 (all concentrations in particles/ml), R is the overflow rate from C_2 for the sample's duration and r_a and r_b are the volume input rates to C_2 from H_2 and the mussel, respectively ($r_a + r_b = R$, all flow rates in ml/min).

E% is then calculated as:

$$E\% = (1 - [C_2]'/[C_1] \times 100 \qquad (2)$$

where $[C_1]$ is the particle concentration in chamber C_1. C_1 overflow was also analyzed for 3.4- to 8.4-μm-diameter particles to give what we refer to as food concentration.

Sampling regimes

In regime A, seven mussels were tested individually for 4 hr at each of the following food concentrations (cells/ml): 3.0×10^3, 1.0×10^4, 2.0×10^4, and 9.0×10^4. Estimates of E% were made every 15 min. Pumping rate, R_p (the rate of water flow through the gill in l/hr), was recorded continuously. The order in which feeding concentrations was presented to the mussels was varied.

Four mussels were observed in regime B, which employed food concentrations of: 2.0×10^4, 9.0×10^4, and 1.1×10^5 cells/ml, presented to mussels in that order. Mussels were kept at each concentration for 2 hr during which four to six estimates of E% were made.

Mussels used in this study were collected sublittorally at Spanish Ship Bay, Nova Scotia, Canada. Acclimation to laboratory conditions (15° C) and continuous feeding were ensured. Mussels ranged in length from 4.5 to 6.0 cm.

Errors in E% estimates

Particle concentrations (by numbers or volumes) in a discrete size category can change because of aggregation, and a potential error exists in interpreting such changes as retention by the gill. Although aggregation of healthy, non-colonial diatoms is unlikely (personal observation), its possible occurrence was tested as follows. Water samples like those normally collected to estimate E% were analyzed for their particle size spectra using a model T_{AII} coulter-counter with a 100-μm tube. Particle concentrations (by numbers and volumes) were estimated in 14 size categories for a total size range of 1.78 to 45.3 μm, each category having a mean volume two times larger than the preceding. The test involved several mussels at various feeding concentrations.

A potentially more significant error could result from the resuspension of feces. Hildreth (in press) estimated that a single mussel can produce 10^7 particles/hr (3.4–8.4 μm) when offered algal concentrations (*Phaeodactylum tricornutum* and *Tetraselmis suecica*) at 10^4 cells/ml, and that levels of fecal resuspension and feeding concentration are directly proportional. Of the food types tested by Hildreth (in press), *Thalassiosira pseudonana* gave least contamination in the 3.4- to 8.4-μm size range, and this diatom was used throughout the present study. However, Hildreth (in press) estimated that mussels fed *T. pseudonana* at a feeding concentration of 4×10^4 cells/ml could produce up to 2×10^6 particles/hr. This figure is used in the present report as an approximate correction factor for estimates of E% at feeding concentrations above 4×10^4 cells/ml.

RESULTS

The data collected in regime A (Fig. 2) show that mean gill-retention efficiency (for seven mussels) for 4.4- to 6.4-μm-diameter particles remains virtually constant as suspension density increases, even when mussels produce pseudofeces. Mean

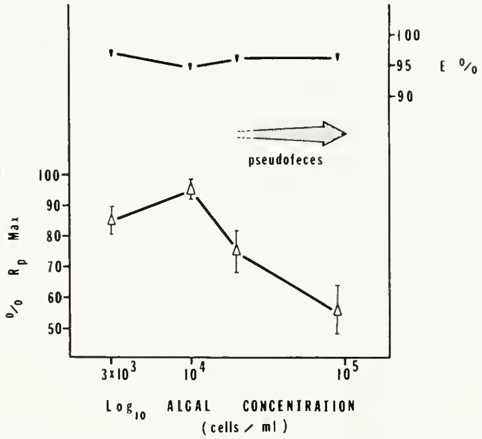

FIGURE 2. Gill retention efficiency (E%) and % maximum pumping rate (% R_p max) as a function of suspension density. Bars on R_p max values are standard errors. Standard errors on E% values are < 1%.

E% from regime B (four mussels) also remained virtually constant with increasing suspension density and was 97.5% at 1.1 × 10^5 cells/ml. At densities above 10^4 cells/ml, reported E% values have been increased by 1% to compensate for errors introduced by fecal resuspension (see Materials and Methods). Examination of the particle size distributions of inhaled and exhaled water suggested no interference by aggregation.

The decrease in pumping rate, R_p, between cell concentrations of 10^4 and 9 × 10^4 cells/ml (Fig. 2) is due either to a decreasing fraction of the total ostia being used, an observation made by Foster-Smith (1974), or to a reduction in lateral ciliary activity. The latter possibility could be due to interference with the cilia by increasing quantities of particulate matter in the ostia or, as Rubenstein and Koehl (1977) have pointed out, because the latero-frontal cirri become so clogged that there is an increasing resistance to flow. The mean pumping rate recorded at 10^4 cells/ml was 5.6 l/hr (for a mean dry flesh weight of 0.70 g).

DISCUSSION

The present study shows that retention efficiency of 5.0-μm diatoms for *Mytilus edulis* remains constantly high over a wide range of particle concentrations. This differs from the results of Davids (1964). However, Davids may have incorporated an artifact into his experimental design, since the hydrostatic pressure in his chamber I was greater than in II (symbols from his Figure 1). Thus when the mussel opened, unfiltered water could pass from I to II through any leak in the mussel/sleeve attachment. Hildreth (1976) has shown how such leaks can be of quantitative importance if a hydrostatic imbalance exists between the chambers. In addition, at low suspension densities, the relatively high pumping rate of Davids' mussel would tend to counterbalance the hydrostatic pressure difference between chambers, thus minimizing the flow of unfiltered water from I to II. At high suspension densities, however, a decrease in pumping would have less countereffect on the flow of unfiltered water, hence an observed inverse relationship between E% and suspension density.

The capacity to vary the retention efficiency of a filtering organ would clearly be of value to a filter feeder. The organism could, for instance, respond passively to high suspended loads in its environment (*i.e.*, by not filtering but still respiring) and minimize the energy expended in rejecting unwanted material. If a filter feeder acts simply to maximize its energy gain from the environment (Lehman, 1976), one would expect an organism which controlled its filter efficiency to possess some advantage over an organism with no control. Taking the argument further, one might expect the effective filter efficiency (*i.e.*, the filtration rate in g of material filtered per unit time) to correlate with optimum ingestion rate. Thus, reported variance in retention efficiencies for a specific size of particle are appealing. However, as Doyle (1979) has pointed out, natural selection may have put constraints on feeding behavior other than those imposed by energy maximization principles. Bivalve filter feeders are clearly an example of this point, since the gill is a complex organ in which the water current is produced within the ostial channel by the simple lateral cilia, but filtration is performed on the gill surface by latero-frontal cirri. An active response to high suspended loads is thus demonstrated by *M. edulis* since, as opposed to a feeding function, the latero-frontal cirri have a major gill-cleansing function in guarding the entrance to the ostial channel.

The gill is the major respiratory organ in the mantle cavity, since it is responsible for producing the ventilation current and probably for much of the gas exchange. The efficiency of oxygen removal for the whole animal is between 5 and 10% under normal conditions but can rise to at least 35% during environmental hypoxia (Bayne, 1971). It would be interesting to see if the efficiency of oxygen removal increases in high suspension densities, an effect perhaps difficult to achieve if the latero-frontal cirri allowed high particulate levels in the ostial channel. Widdows *et al.* (1979), using resuspended, naturally occurring particles, have observed a significant increase in oxygen extraction efficiency with increasing particle concentration. The tips of lateral cilia (15 μm long) of adjacent filaments may touch during their beat cycle (Sleigh, 1974), and so particulate matter in the ostial channel presumably interferes with the coordination and power output. Widening of the ostial channel by muscular and vascular action could act to reduce such interference but probably not by much, according to the dimensions quoted by Dral (1967) and Foster-Smith (1976).

Inferences concerning the coordination of the latero-frontal cirri can be made by comparing E% and R_p for individual mussels that show enough variation in R_p to make this feasible. Figure 3 indicates that the majority of mussels tested exhibit cirral coordination over the range of pumping rates observed.

Moore's (1971) and Owen's (1974) estimate of a minimum mesh dimension of 0.6 μm is only valid if adjacent cirri on the same filament are asynchronous by half a beat. Examination of Owen's (1974) Figure 17 shows that one synchronous pair of cirri would produce a "hole" of approximately 2 μm in the filter meshwork. One and a half or two synchronous pairs would locally increase the minimum mesh dimension to 4 or 6 μm, and so on. The phenomenon of synchrony in adjacent cirri would give a significant variance to the filter's minimum mesh dimension, which could explain the decreasing percentage capture of particles < 2 to 4 μm observed for the macrociliobranchia (Haven and Morales-Alamo, 1970; Vahl, 1973) and also the low retention (about 86%) of 4.4- to 6.4-μm particles occasionally observed in the present study (Fig. 3). Irregular spacing between cirri (Dral, 1967) and a length of 1.4 μm between distal branches of the

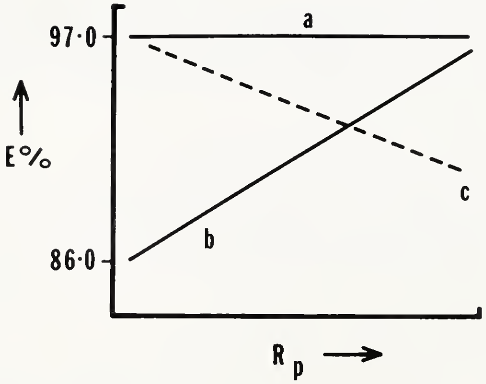

FIGURE 3. Diagrammatic representation of the relationship between E% and R_p, based on individual data for nine mussels fed at 9×10^4 cells/ml. For type a) behavior (shown by six of the mussels tested), latero-frontal cirral activity appears to be coordinated (*i.e.*, alternate cirri are in phase). Types b) and c) behavior (each shown by 1 mussel) indicate degrees of cirral uncoordination (*i.e.*, $\geqslant 3$ adjacent cirri become synchronous or stop beating in a halt position). One mussel showed a b)/c) combination. Types b) and c) behavior were not frequent enough to lower the mean E% values given in Figure 2.

cirrus (Jorgensen, 1975) would also increase the variance in minimum mesh dimension. When considering the whole gill surface, it is probable that water flows preferentially through the larger holes due to decreased drag. Although considered infrequent, synchrony of adjacent cirri could thus cause significant decreases in retention efficiency. Observed retention efficiencies can therefore be explained by the sieving action of the cirri, and the alternate mechanisms of particle capture proposed for mussels by Rubenstein and Koehl (1977) may not apply. The situation here is somewhat analagous to that commented on by Boyd (1976). Boyd suggests that reported retention efficiencies of copepods' second maxillae are a function of the significant variance in mean intersetule distance. Reported retention efficiencies for copepods can then be explained by the physical properties of the filter mesh rather than by particle size-selection behavior by the animal.

The observations of Jorgensen (1975) concerning the effects of serotonin solutions on the porosity of the gill are difficult to interpret with respect to the natural situation. Many workers assume 100% retention efficiency of circa-5-μm particles in order to estimate filtration rates. And many have claimed to have shown 100% retention of such particles. Although we observed a constant high retention efficiency, further investigation is clearly necessary. Care in interpretation of retention observations should be exercised, however, because *M. edulis* is known to give rise to bioseston (Vahl, 1972; Hildreth, in press); and escape of smaller particles is likely since cirral branches have stiffening rods which extend distally less than half way up their length (Owen, 1974).

For 4.4- to 6.4-μm particles, the observations made by Dral (1967) concerning *Mytilus edulis* are not as quantitatively significant as he supposed. We conclude that the continuous meshwork formed by the latero-frontal cirri does not allow the gill to become porous to this particle size, even at high suspension densities when the rejection of filtered material could become energetically costly. It seems to be more expedient for a mussel to keep its ostia clear. However, the variations in cirral beat described by Dral (1967) and Owen (1974) could explain the empirical data on the gill's retention efficiency for smaller particles than were examined in the present report. It is intended that this aspect will be the basis for a further study.

SUMMARY

1. The retention efficiency, E%, of the *Mytilus edulis* gill for 5.0-μm diatoms (*Thalassiosira pseudonana*) was estimated by separating the bivalve's exhalant current through a rubber sleeve attached to the valves around the exhalant siphon.

2. The particle concentration in the 4.4– to 6.4-μm size range of inhaled and exhaled water was estimated with a model Z_B coulter-counter. The mussel's pumping rate, R_p, was recorded automatically.

3. E% and R_p were observed for 11 mussels at diatom concentrations ranging from 3×10^3 to 1.1×10^5 cells/ml. R_p was maximal at 10^4 cells/ml and at 9.0×10^4 cells/ml decreased to about 55% of its maximum value. E% remained constantly high (about 97%) over the range of concentrations even when pseudofeces (filtered material rejected before ingestion) were produced. This result supports the argument that, in general, a continuous filtering meshwork is formed over the ostia by synchrony in alternate (or alternate pairs of) latero-frontal cirri on the same gill filament.

4. Occasionally, a low E% value (about 86%) was observed for an individual mussel and this was attributed to synchrony in $\geqslant 3$ adjacent cirri.

5. Synchrony in one or more pairs of adjacent cirri is advanced as an explanation for published data on the retention of different sized particles.

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MORPHOGENIC MOVEMENT AND EXPERIMENTALLY INDUCED DECREASE IN NUMBER OF EMBRYONIC SEGMENTS IN THE JAPANESE HORSESHOE CRAB, *TACHYPLEUS TRIDENTATUS*[1]

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There have been several intensive studies on the normal embryonal development of horseshoe crabs (Packard, 1870, 1872, 1880; Kingsley, 1892, 1893; Kishinouye, 1892; Munson, 1898, 1912; Patten, 1912; Gardiner, 1927; Iwanoff, 1933; Brown and Knouse, 1973; Sekiguchi, 1973; Bennett, 1979), and a few experimental studies of the dense egg masses (Patten, 1894, 1896), by electrocauterization (Oka, 1943; Sekiguchi, 1966) and by cell dissociation (Itow and Sekiguchi, 1979; Itow, 1979). However, the detailed process of the morphogenic movement has remained unrevealed.

In this study, the process of morphogenic movement in the embryo of the horseshoe crab was examined with time-lapse cinemicrographic and histological techniques. In addition, embryos were treated with chemical reagents such as cytochalasin B and dithiothreitol to attempt further elucidation of normal morphogenic movement. In the course of the experiment, monsters showing decrease in number of segments were elicited. Therefore, the characteristics, conditions of induction and process of formation of those monsters were investigated.

The present paper describes the process of morphogenic movement, the induction of monsters, and the relationships between them.

MATERIALS AND METHODS

Adult Japanese horseshoe crabs, *Tachypleus tridentatus,* collected at the beach at Imari, Saga Prefecture, and Kasaoka, Okayama Prefecture, were brought to the Shimoda Marine Research Center of the University of Tsukuba, Shizuoka Prefecture. There, eggs were artificially inseminated and the fertilized eggs reared in filtered normal sea water in plastic trays at room temperature (24–32° C). The sea water in the trays was exchanged for fresh sea water once or twice a day. The embryos used for observations and experiments were taken from these trays.

The stages of embryonic development were identified by reference to the normal stages described by Sekiguchi (1973).

Cinemicrography

Normal and treated embryos vitally stained with neutral red were filmed using a Nikon 16 mm cinemicrographic apparatus (type CFM–A) at intervals of 1–8 min (usually 4 min). The movements of various parts of the embryos were analyzed by tracing them on the serial cinefilms.

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[1] Contributions from the Shimoda Marine Research Center, No. 354.

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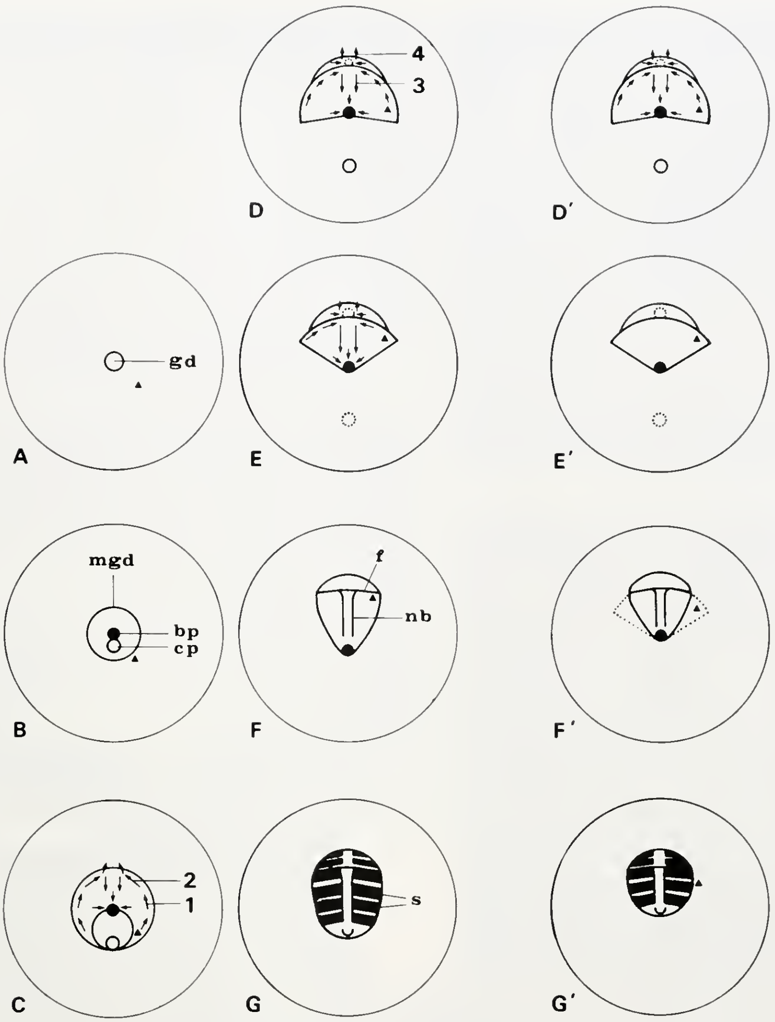

FIGURE 1. Diagrammatic representation of morphogenic movement in normal and segment-defective embryos, drawn from the cinemicrographs.

A. to G. Key morphogenic events in the normal embryo:

A. Germ disc appearance (Stage 7),
B. Spreading of the germ disc (Stage 9),
C. Germ disc completion (Stage 10),
D. Half a day after Stage 10,
E. A day after Stage 10 (Stage 11),
F. End of morphogenic movement (Stage 12),
G. Appearance of segment structure (Stage 13).

D′. to G′. Morphogenic movement in the embryo treated with chemical reagents for 24 hr from stage D.

D′. The stage just after treatment.

Arrows show direction of migration of the surface cells. Solid triangles show points of surface cells traced by cinemicrography. The explanation of 1 to 4 is in the text. bp: blastopore, cp: cumulus posterior, f: region of transverse furrow, gd: germ disc, mgd: margin of germ disc, nb: narrow band, s: segment structure.

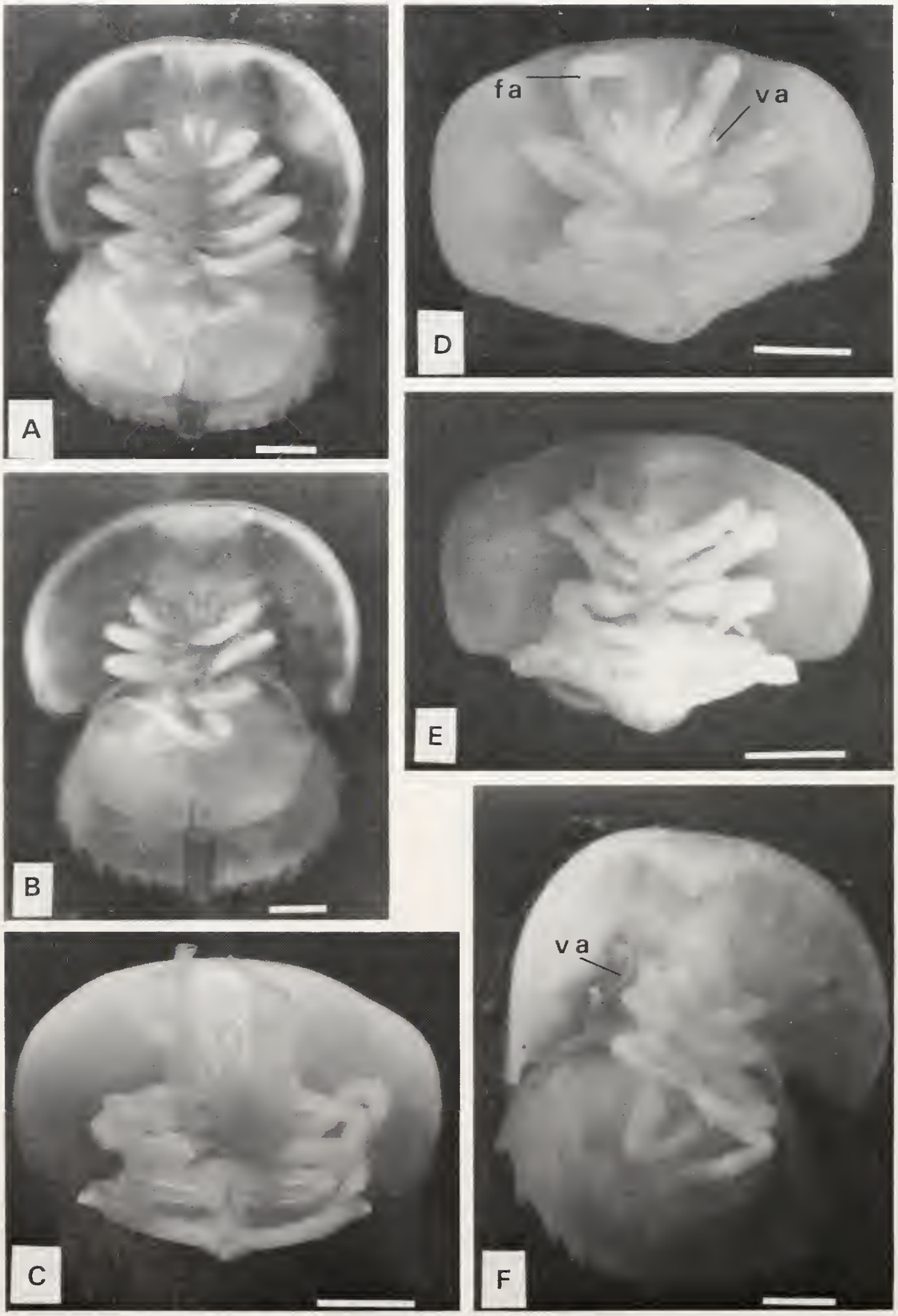

Figure 2. Examples of segment-defective embryos.
A. A normal embryo with six pairs of prosomal appendages.
B. A typical segment-defective embryo devoid of the 3rd prosomal segment.

Chemical and physical treatments

Chemical reagents. Many groups of 20 embryos each were cultured separately in small laboratory dishes filled with about 10 ml of natural or artificial sea water containing cytochalasin B (Cyt. B) dissolved in dimethylsulfoxide, or dithiothreitol (DTT) dissolved in distilled water. Other embryos were treated with mercaptoethanol, o-iodosobenzoic acid dissolved in dimethylsulfoxide, $HgCl_2$, arsenite, α-lipoic acid, α-lipoic amide, colchicine, vinblastine sulfate, dimethylsulfoxide and Ca^{2+}-free sea water, respectively. Treatment time varied with the purpose of the experiment. During treatment, the culture media were maintained between 28° and 30° C and renewed every 12 hr. After treatment, the embryos were returned to normal sea water which was also renewed every 12 hr.

Temperature. Embryos at selected stages were exposed to 5°, 37°, or 50° C to observe the effects of low or high temperature on morphogenesis.

Electrocauterization. Specific portions of the embryos were electrically cauterized during morphogenic movement by the method of Oka (1943).

Light and electron microscopy

Normal embryos and embryos treated by the various methods mentioned above were vitally stained with neutral red and observed under a stereoscopic microscope. The histological observations were made by the following light and electron microscopic methods:

1. For light microscopy, normal and treated embryos were fixed in Bouin's, Carnoy's, or FAA (formalin-ethanol-acetic acid, 5 : 5 : 1) solutions, embedded in paraffin, and sectioned at 5–20 μm. The sections were stained with Mayer's hematoxylin and eosin, or Azan staining solution.
2. For electron microscopy, specimens were pre-fixed in 5% glutaraldehyde and 4% paraformaldehyde in 8×10^{-2} M cacodilate buffer (*p*H 7.4) for 12 hr, and then post-fixed in 1% osmium tetroxide solution for 2 hr. The sections were stained with saturated uranyl acetate and Reynold's solution.

RESULTS

Morphogenic movement

Migration of cells in the embryo. The results obtained by time-lapse cinemicrography and stereoscopic microscopy are summarized as follows:

The germ disc appeared as a spot on the blastoderm at Stage 7, about 8 days after insemination (Fig. 1-A). The germ disc increased gradually in diameter and reached a maximum at Stage 10 (Fig. 1-C), the stage of germ-disc completion,

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C. A segment-defective embryo with right-hand appendages normal, but the 1st to 4th appendages fused on the left side.

D. A segment-defective embryo, with fused 2nd and 3rd appendages and vestigial 4th ones on both sides.

E. A segment-defective embryo obtained by treatment with dithiothreitol for 48 hr. The abnormal region is wide and the 1st to 4th appendages show some abnormalities such as defect or fusion.

F. A segment-defective embryo with a vestigial right 3rd appendage not in line with the five pairs of normal ones.

The embryos of A, B and F were photographed after the 4th embryonic moulting and the embryos of C, D and E after the 3rd embryonic moulting. White bar = 1 mm. fa: fused appendage, va: vestigial appendage.

about 12 days after insemination. During this process, the so-called blastopore appeared at the central portion of the germ disc and the cumulus posterior that appeared at Stage 9 near the blastopore migrated to the inner margin of the posterior end of the germ disc (Fig. 1-B).

Obvious morphogenic movement started in Stage 10. The germ disc began to change its shape. The surface cells of the whole germ disc began to migrate actively. Main migrations of the cells, occurring simultaneously, were as follows (refer to 1–4 in Fig. 1-C and D) : 1.) The cells in the lateral region of the peripheral area of the germ disc migrated to the anterior region. 2.) The cells in the lateral region of the anterior area of the germ disc migrated towards the median body axis of the embryonic area. 3.) The major portion of the cells at the anterior margin of the germ disc moved posteriorly along the median body axis, forming two narrow bands (Fig. 1-F). 4.) Some cells of the anterior margin of the germ disc spread in front of the germ disc.

During these migrations, the blastopore moved to the posterior end of the embryonic area. The embryonic area stretched to double its original length. A transverse furrow was formed at the anterior region of the embryonic area during morphogenic movement. The region where this furrow appeared was identical to the anterior margin of the germ disc at Stage 10 (Fig. 1-C).

Morphogenic movement continued for about two days until Stage 12 (Fig. 1-F), being most rapid at Stage 11 (Fig. 1-E). Segment structures appeared on the embryonic area at Stage 13 (Fig. 1-G). First, the 3rd prosomal segment appeared clearly at the region immediately behind the furrow of the embryonic area, where the peripheral cells of the germ disc gathered actively. The 1st and 2nd prosomal segments appeared in front of this region, and the 4th and the 5th ones appeared behind the 3rd segment. However, the 6th prosomal segment and the opisthosomal segments were not so clear in this stage.

Histological and cytological changes. Some cells larger than the blastoderm cells appeared under the germ disc at Stage 7. These cells, which are not identical with yolk cells, divided actively and began to form the underlayer of the germ disc. After Stage 7, the surface cells of the germ disc changed from flat to cubic, and formed a more compact layer than the other surface cells of the embryo. The reduction in size of the surface cells both inside and outside the germ disc continued uninterrupted from the stage of cleavage on. From insemination to Stage 10, each nucleus of the cells of the surface and the underlayer usually had two nucleoli.

During active morphogenic movement (Stages 10 to 12), the following changes were observed : The cells of the underlayer became smaller and increased in number, and, at the anterior region of the embryonic area, two bands of cells were formed along the median body axis. The surface cells of the embryonic area became cylindrical and more compact. This transformation was especially distinct in the anterior region of the embryonic area. The reduction in size of the cells of the underlayer and surface ended during morphogenic movement. The number of nucleoli in each nucleus decreased, usually from two to one. At Stage 12, microfilaments were observed in the periphery of surface cells at the level of apical junction.

Formation of segment-defective embryos

When the embryos were treated for 24 hr with 5–10 μg/ml Cyt. B or 300–5000 μg/ml DTT during the period of most active morphogenic movement, more than

TABLE I

Frequencies of formation of segment-defective embryos and other monsters by treatment with dithiothreitol and cytochalasin B for 24 hr during active morphogenic movement.

Reagents	No. of normal embryos (%)	No. of segment-defective (%)	No. of other monsters (%)	No. of developed embryos (%)	% of developed embryos per total embryos
Dithiothreitol 500 μg/ml	54 (27.14)	142 (71.36)	3 (1.50)	199 (100.00)	71.1
Cytochalasin B 5 μg/ml	7 (21.21)	26 (78.79)	0 (0.00)	33 (100.00)	66.0
Normal sea water (for control)	2539 (99.57)	1 (0.40)	10 (0.39)	2550 (100.00)	84.3

50% of them formed monsters (Fig. 2, Table I). External and histological observations of these monsters revealed that the defect or fusion of appendages occurred with a simultaneous defect or fusion of the corresponding segments.

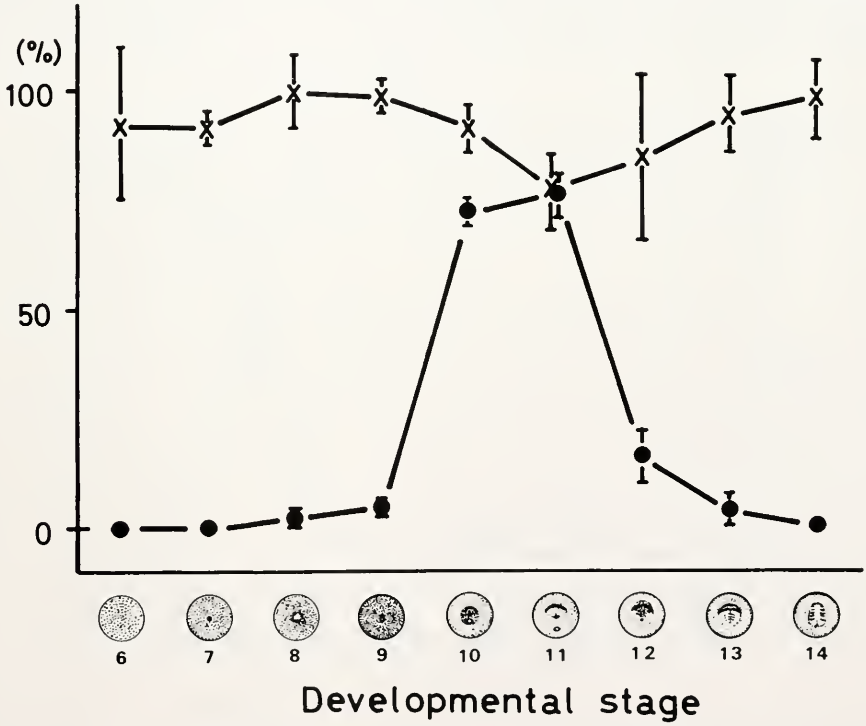

FIGURE 3. Rates of development and formation of segment-defective embryos after 24 hr treatment with dithiothreitol at different developmental stages. Cross: mean rate of development in the treated group as a percentage of the rate in controls. Solid circle: mean rate of formation of segment-defective embryos among developed embryos. Vertical lines with limiting marks: ± 1 standard deviation. The embryos were treated at stages shown in the figure.

These monsters are called segment-defective embryos hereafter. Segment-defective embryos were also induced at a rate higher than 50% by treatment for 24 hr during morphogenic movement with 1.5–3 μg/ml arsenite, 1,300 μg/ml to saturated o-iodosobenzoic acid, 5 μg/ml to saturated α-lipoic acid, 5 μg/ml to saturated α-lipoic amide or 1–2 μg/ml $HgCl_2$. After treatment of the embryos with 7,500–15,000 μg/ml mercaptoethanol, segment-defective embryos were formed at a rate of 0–50%.

The conditions for formation of segment-defective embryos were as follows:

1. Developmental stage. Treatment with the above-mentioned chemical reagents (except Cyt. B) induced segment-defective embryos at any stage during morphogenic movement (Fig. 3). When embryos at Stage 10 were treated with Cyt. B, the death rate became higher and few segment-defective embryos were induced (Fig. 4). With this reagent, monsters were induced only when the embryos were treated at Stage 11 (1 day after Stage 10). When the embryos were treated with the above-described reagents only before or after morphogenic movement, segment-defective embryos were not induced.

2. Treatment time. Embryos treated with the above-mentioned chemical reagents for 24 hr formed segment-defective embryos at the highest rate. They were formed at nearly the same rate with treatment for only 12 hr at Stage 11.

3. Concentration of chemical reagents. When treated for 24 hr with DTT during morphogenic movement, embryos treated with less than 200 μg/ml DTT developed normally and embryos treated with more than 10,000 μg/ml DTT

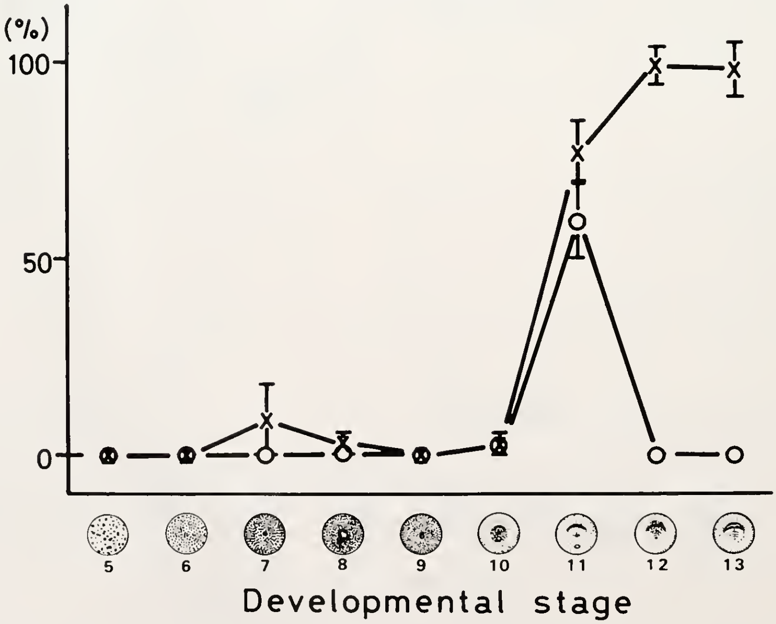

FIGURE 4. Rates of development and formation of segment-defective embryos after 24 hr treatment with cytochalasin B at different developmental stages. Cross: mean rate of development in the treated group as a percentage of the control rate. Open circle: mean rate of formation of segment-defective embryos as a percentage of developed embryos in the control experiment. Vertical line with limiting marks: ± 1 standard deviation. Embryos were treated at the stages shown in the figure.

stopped developing. Concentrations between these caused segment-defective embryos. When embryos at Stage 10 were treated with Cyt. B for 24 hr, embryos treated with more than 5 μg/ml Cyt. B did not develop and those treated with less than 2 μg/ml Cyt. B developed normally. With treatment for 24 hr with Cyt. B at Stage 11, embryos treated with less than 2 μg/ml Cyt. B developed normally and embryos treated with more than 20 μg/ml Cyt. B did not develop. Concentrations between these caused segment-defective embryos.

4. Other reagents. When embryos were treated with Ca^{++}-free sea water for 24 hr during morphogenic movement, various types of monsters were formed. Segment-defective embryos were included in these, but the rate was lower than 25% of developed embryos. Colchicine, vinblastine sulfate, and dimethylsulfoxide failed to induce segment-defective embryos or any other monsters. That is, when embryos were treated for 24 hr during morphogenic movement with less than 10^{-1} μg/ml colchicine, less than 1 μg/ml vinblastine sulfate or less than 5 μg/ml dimethylsulfoxide, they developed normally. When embryos were treated for 24 hr during morphogenic movement with more than 1 μg/ml colchicine or 10 μg/ml vinblastine sulfate, they did not develop.

5. *Temperature.* Treatment with low or high temperature failed to produce segment-defective embryos. When embryos were cultured for 24 hr at 5° or 37° C during morphogenic movement, they developed normally. With treatment at 50° C, embryos did not develop.

Characteristics of segment-defective embryos

The segment-defective embryos obtained by treatment with chemical reagents such as Cyt. B and DTT showed the following characteristics: (No appreciable difference was recognized between the effects of the different reagents.)

Most of the segment-defective embryos showed an unsymmetrical defect or fusion of appendages.

Fusion was observed between the anterior and the posterior appendages of the right or left side, but not between the right and the left sides.

The occurrence of abnormal appendages is shown in Figure 5. About 50% of the examined sides having abnormal appendages showed the defect in only one appendage. As the appendages between the 2nd and the 5th have similar forms, the difference between appendages is not clear at first sight and the appendage cannot always be confirmed. Therefore, the structure and size of the appendages of segment-defective embryos with five pairs of prosomal appendages were compared with those of normal embryos. It was revealed that the 3rd prosomal appendages in these monsters disappeared and the other appendages were hardly different from normal ones (Fig. 6). In addition, it was found that some embryos having only four pairs of prosomal appendages were devoid of the 3rd and the 4th appendages.

In some of the segment-defective embryos, a vestigial 3rd prosomal appendage was observed outside the normal row of appendages (Fig. 2-F).

In cases of fused appendages, the fusion between the 3rd and the 4th prosomal appendages was most frequent, followed by fusion between the 2nd and the 3rd appendages.

In all segment-defective embryos, the 5th and 6th prosomal appendages as well as all the opisthosomal ones were always normal (Fig. 5).

From these results, it may safely be said that abnormality of appendages occurs mainly in the 3rd prosomal segment.

Relationships between the conditions of induction and the types of abnormality of appendages were as follows:

No significant difference was observed in the types of defects following treatment at different developmental stages.

With treatment for less than 24 hr, embryos devoid of one appendage were fewer and embryos with one or two fused appendages were more common, compared with 24-hr treatment. When treatment time was longer than 24 hr, the abnormal area became wider and various types of defects and fusion were observed in the 1st to the 4th prosomal appendages (Fig. 2-E).

When embryos were treated with lower than 500 μg/ml DTT or 5 μg/ml Cyt. B, embryos having fused appendages outnumbered those devoid of one appendage. In concentrations higher than 500 μg/ml DTT or higher than 5 μg/ml Cyt. B, embryos lacking one appendage on one or both sides outnumbered embryos with fused appendages. With these treatments, however, the abnormality of appendages was limited almost entirely to the 3rd prosomal segment, and the abnormal area did not become wider.

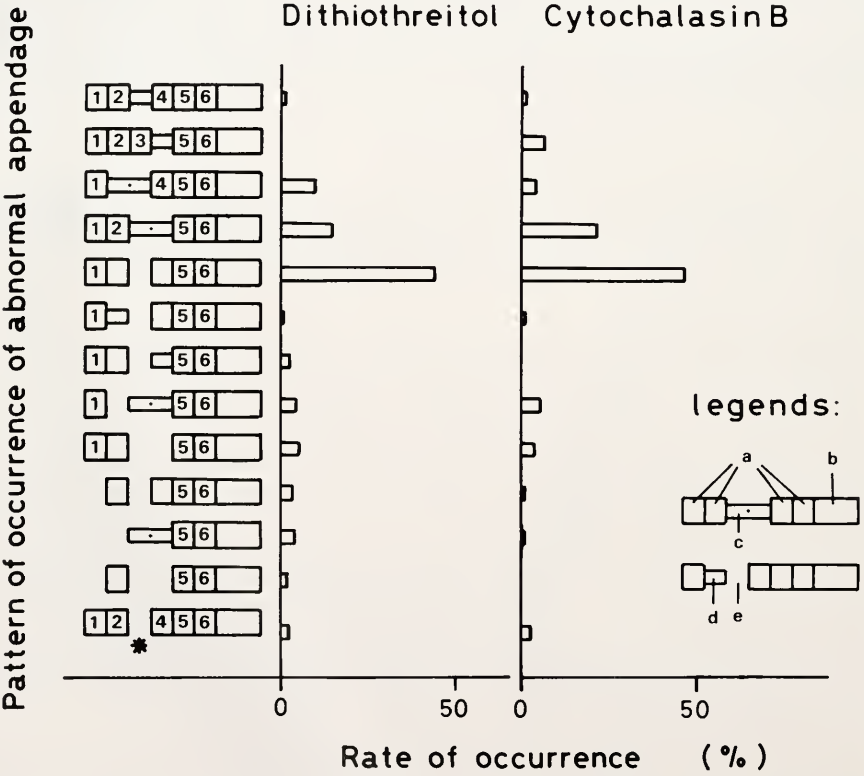

FIGURE 5. The pattern and rates of occurrence of abnormal appendages induced with dithiothreitol and cytochalasin B. a: normal prosomal appendages. b: normal opisthosome (abdomen). c: a vestigial appendage. d: fused abnormal appendages. e: missing appendages. But the 2nd to 5th appendages of the horseshoe crab embryos have similar forms, so the missing appendages are not always clear. *: the pattern showing a vestigial 3rd prosomal appendage not in line with other appendages. 1 to 6: These numerals show the appendages having the same structure as the 1st to 6th prosomal ones of normal embryos.

Characteristics of morphogenesis in embryos treated with reagents

When embryos were treated with chemical reagents effective in inducing segment-defective embryos, morphogenic movement stopped almost completely during treatment (Figs. 1, 7). When these embryos were returned to normal sea water, morphogenic movement started again. However, both migration of the peripheral cells of the germ disc and elongation of the embryonic area ended without their attaining normal position and length, and the segment structures appeared successively. These embryos developed into embryos showing abnormality in or near the 3rd prosomal segment.

Morphogenic movement also stopped during exposure to low temperature (5° C). However, these embryos developed normally when returned to an incubator at 28°–30° C. Morphogenic movement and the elongation of the embryonic area started again and proceeded normally.

Histological observations revealed phenomena common to embryos treated with chemical reagents and those cultured at low temperature. In both, the cells in the underlayer were prevented from becoming smaller, increasing in number, or gathering. In addition, the transformation of the surface cells of the embryo into cylindrical shape was stopped. However, the decrease in the number of

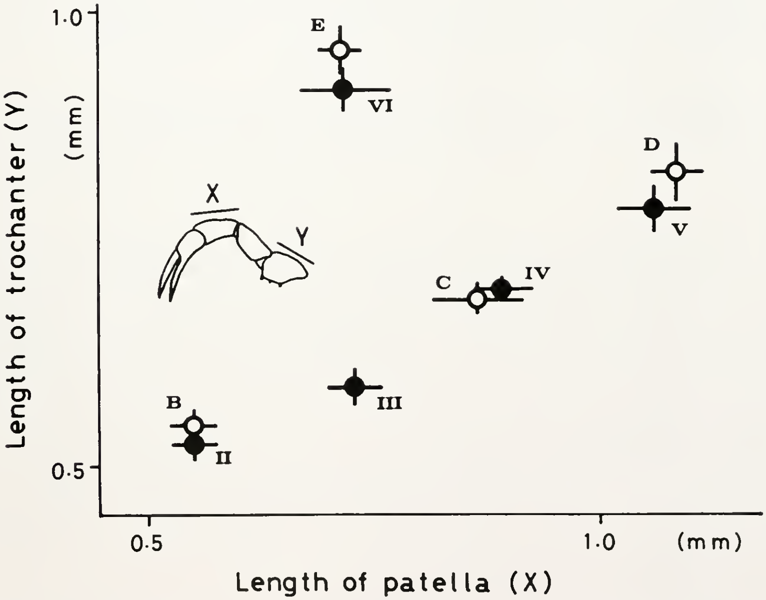

FIGURE 6. Comparison of the length of two portions of the appendages in normal embryos and segment-defective ones having five pairs of prosomal appendages at hatching stage. (Each appendage of 30 normal embryos and 13 segment-defective embryos was cut out and measured with micrometer under the microscope. The unit of measurement was 10 or 25 μm.)

Closed circle: normal embryo. Open circle: segment-defective embryo. Vertical and horizontal bars: ± 1 standard deviation.

II to VI: 2nd to 6th prosomal appendages in the normal embryo. B to E: 2nd to 5th prosomal appendages of segment-defective embryos from anterior to posterior. The 5th appendages or the last prosomal appendages of these defective embryos have the same structure as the 6th ones or the last prosomal ones of normal embryos. In short, this type of segment-defective embryo lost only the 3rd prosomal segment.

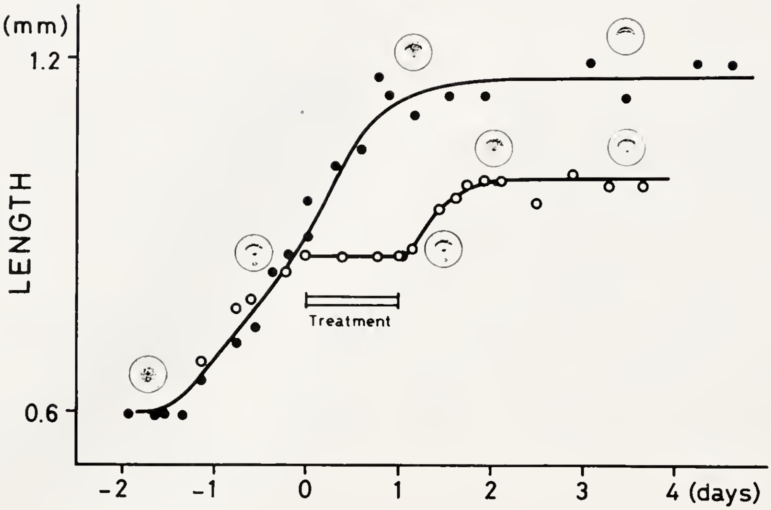

FIGURE 7. Elongation of the embryonic area observed by cinemicrography in normal and treated embryos. Closed circle: length of embryonic area in normal embryos. Open circle: length of embryonic area in an embryo treated with dithiothreitol for 24 hr at the period shown. The treated embryo developed lacking the 3rd prosomal appendage in the right side and with fused 3rd and the 4th appendages on the left. Length of embryonic area was measured between the anterior end and the blastopore.

nucleoli differed between these two treatments. The number of nucleoli decreased with treatment by chemical reagents, but not with culture at 5° C. When embryos were treated with chemical reagents, no enucleated or polynucleated cell was observed.

By electron microscopic observations, a difference in the adhesion of surface cells was recognized between normal embryos and embryos treated with chemical reagents. In normal embryos, the cells showed complete adhesion. However, in embryos treated with DTT or Cyt. B, adjacent cells were partly separated. Microfilaments were observed at the apical circumferences of the cylindrical surface cells in the normal embryo, but they could not be observed in the cubic surface cells of the normal embryo or in the cubic cells whose transformation into cylindrical shape was stopped with chemical reagents. On the other hand, microtubules were observed even in the treated embryos. In embryos treated with chemical reagents, secretion of the inner egg membrane and transformation of the surface cells were stopped in the same state as before treatment. Organelles such as mitochondrion, Golgi body, ribosome, nucleus and cell membrane showed no recognizable difference between the normal and the treated embryos.

By histological observations, no appreciable difference in histological features was recognized between hatched larvae cultured in normal sea water after being returned from the treatment solution and normal larvae, although a slight retardation of development was observed in the treated embryos.

Formation of segment-defective embryos by electrocauterization

Segment-defective embryos were also formed by electrically cauterizing the same region in which cell migration to the normal position was inhibited by chemical treatment.

The position indicated by the black triangle in Figure 1-E and the adjacent region were cauterized in 57 embryos. Of these 57 embryos, 34 developed. Fourteen of them were segment-defective embryos. The defects and fusion were observed at the 3rd prosomal segment and the neighboring segments in the left side of embryos. The abnormal segments were much the same as those in the monsters obtained by treatment with chemical reagents.

DISCUSSION

It has been known that in some Vertebrata and Echinodermata morphogenic movement can be disturbed by chemical reagents such as Cyt. B and DTT, and that various types of malformations such as incomplete formation of archenteron and neural tube are induced (Runnström and Kriszat, 1952; Runnström, 1956; Brachet, 1959; Seilern-Aspang, 1959; Laskshmi, 1962; Wessells *et al.,* 1971; Schaeffer and Brick, 1972; Brachet and Tencer, 1973).

In the present study, the authors observed the processes of morphogenic movement and histological changes of the embryonic area during morphogenic movement in certain developmental stages of the horseshoe crab (Fig. 1). The study revealed that morphogenic movement of the horseshoe crab can be disturbed by treatment with Cyt. B or DTT, and that more than 50% of the treated embryos develop into segment-defective embryos (Table I; Fig. 2). Mercaptoethanol, o-iodosobenzoic acid, arsenite, $HgCl_2$, α-lipoic acid and α-lipoic amide also caused segment-defective embryos at a high rate. This implies that such reagents prevent embryos from completing morphogenic movement, since they are generally thought to be inhibitors of cell movement via disturbance of SH-SS interchange or microfilament formation.

The authors tried several kinds of experiments to test this possibility and obtained the following results showing a positive relationship between experimental induction of segment-defective embryos and disturbance of morphogenic movement: The optimal time for experimental induction of segment-defective embryos coincided well with the period of morphogenic movement (Figs. 3, 4). Cinemicrographic observation showed that morphogenic movement ceased completely under treatment with the chemical reagents, and that after treatment it proceeded normally again but stopped before attaining the final state of morphogenic movement in normal embryos (Figs. 1, 7). The segment-defective embryos thus obtained commonly also had lost the 3rd prosomal segment (Figs. 5, 6). The location of the prospective region which takes part in the formation of the 3rd prosomal segment was confirmed by electrocauterization experiments, which obtained the same type of segment-defective embryo as was induced with chemical reagents. Since the 3rd prosomal segment may originate from cells which migrate from the periphery of the germ disc to the region just behind the furrow of the anterior embryonic area, incomplete morphogenic movement probably brings about the exclusion of the cells prospective for the 3rd prosomal segment (Fig. 1). Embryos having a small vestigial 3rd prosomal appendage located outside a normal row of other appendages are thought to be a result of disturbance of morphogenic movement due to the chemical reagent treatment (Fig. 2-F). The defective area became wider with treatment longer than 24 hr. This may be explained by an increase of the excluded area as a result of less complete morphogenic movement.

The mechanism inducing incomplete morphogenic movement has, so far, been unclear. However, we can speculate that incomplete morphogenic movement might arise from dissociation of coordination between cell movement and morphogenesis. In normal development, of course, they would be coordinated. With low-temperature treatment, both may be stopped simultaneously during treatment, and treated embryos may develop normally after they are transferred to 28–30° C. Coordination may be dissociated by chemical reagent treatment. It seems to be evidence of dissociation of coordination that the decrease of nucleolus number was not accordant with morphogenic movement in embryos treated with chemical reagents. If the formation of segment structure begins during the delayed cell movement, the cells of a region could be excluded.

Analysis of experimentally induced segment-defective embryos gives us some information on the mechanism of normal morphogenic movement, segmentation and appendage formation. Body segmentation can occur even when morphogenic movement is incomplete. The 3rd prosomal segment seems not to be necessary for normal development of the other segments or appendages, since other appendages of embryos devoid of the 3rd prosomal appendage had the same structure and size as those of the normal embryo (Fig. 6). However, the region near the furrow of the anterior embryonic area may be important for morphogenesis, since morphogenic movement is most active there. Some appendages and segments may be determined to some extent at stages before morphogenic movement, because, in spite of defects of some segments, other segments and appendages of an embryo form normally. Abnormalities such as defects or fusion of appendages occur independently in the left and the right sides of the same segment-defective embryo. It is likely, therefore, that the migration of cells on each side of the embryonic area and the formation of segments of each side proceed independently to a certain extent.

Morphogenic movement in the horseshoe crab shows the same pattern as seen in the Entelegynae of the Araneida in which "Umkippen" occurs (Holm, 1952). This result is inconsistent with the fate map depicted by Anderson (1973) from the study of Iwanoff (1933) but consistent with the results of Sekiguchi (1973).

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SUMMARY

Active morphogenic movement in the horseshoe crab, *Tachypleus tridentatus,* was observed during the 2 days following completion of the germ disc.

Main migration of cells is as follows: Peripheral cells of the germ disc migrate to the anterior margin of the germ disc, and the cells of the anterior margin of the germ disc migrate posteriorly along the median body axis. During such migrations, the transverse furrow appears at the anterior margin of the germ disc.

The 3rd prosomal segment is formed from the region just behind the furrow.

By treatment of embryos with chemical reagents such as cytochalasin B, dithiothreitol and α-lipoic acid, morphogenic movement was disturbed and segment-defective embryos were formed.

Defect or fusion of appendages and segments in the segment-defective embryos occurred mainly in the 3rd prosomal segment. A possible explanation is that the prospective region forming the 3rd prosomal segment cannot take part in the formation of segments as a result of the inhibition of morphogenic movement by chemical reagents.

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ARTIFICIAL INDUCTION OF OOCYTE MATURATION AND DEVELOPMENT IN THE SEA CUCUMBERS *HOLOTHURIA LEUCOSPILOTA* AND *HOLOTHURIA PARDALIS*[1]

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Although a number of works have been written on the development of holothurians (MacBride, 1914; Korschelt, 1936; Hyman, 1955; Inaba, 1957; Oguro, 1974), extensive experimental studies on development have not been carried out. One reason for this may be difficulties in obtaining mature eggs: selection of mature eggs from excised ovaries or induction of spawning has been practical for only some species of holothurians (Ohshima, 1925a, b; Colwin, 1948; Inaba, 1957; Strathman and Sato, 1968). In two species common on the coast of Japan, *Holothuria leucospilota* and *Holothuria pardalis,* mature eggs are rarely found in excised ovaries and oocytes isolated from ovaries do not mature spontaneously in sea water.

The present paper reports success of artificial maturation of isolated oocytes from ovaries of *H. leucospilota* and *H. pardalis.* With the use of some disulfide reducing agents, nearly all full-grown oocytes of these two species were induced to mature. These mature oocytes formed the first and second polar bodies, and after insemination they developed to typical auricularia larvae.

MATERIALS AND METHODS

Specimens of *Holothuria leucospilota* and *Holothuria pardalis* were collected during a presumed breeding season (July to August) at the coast near the Seto Marine Biological Laboratory of Kyoto University.

Isolated ovaries were washed with sea water three times. Oocytes excised from the ovaries with fine forceps were further washed with sea water three times, and then used for experiments. These isolated oocytes rarely matured spontaneously in sea water. During this season, the oocytes were rather uniform in size. Oocytes of *H. leucospilota* were transparent and pale pink in color and those of *H. pardalis* were transparent and colorless.

Dithiothreitol (DTT) (Wako Pure Chemical Industries and Nakarai Chemicals, Kyoto), 2,3-dimercapto-1-propanol (BAL) (Wako), L-cysteine (Sigma Chemical Co., St. Louis) and glutathione (reduced form) (Wako) were dissolved in sea water just before use. 1-methyladenine (1-MA) (Sigma) also prepared for experiments of oocyte maturation, was dissolved in distilled water at 1×10^{-3} M and diluted with sea water just before use (Kishimoto and Kanatani, 1973).

The subsequent experiments were carried out at the water temperature of 28–29° C.

RESULTS

Induction of oocyte maturation with dithiothreitol (DTT)

The DTT solution (1×10^{-3} M in sea water) induced the breakdown of germinal vesicles of immature oocytes from *H. leucospilota* and *H. pardalis.*

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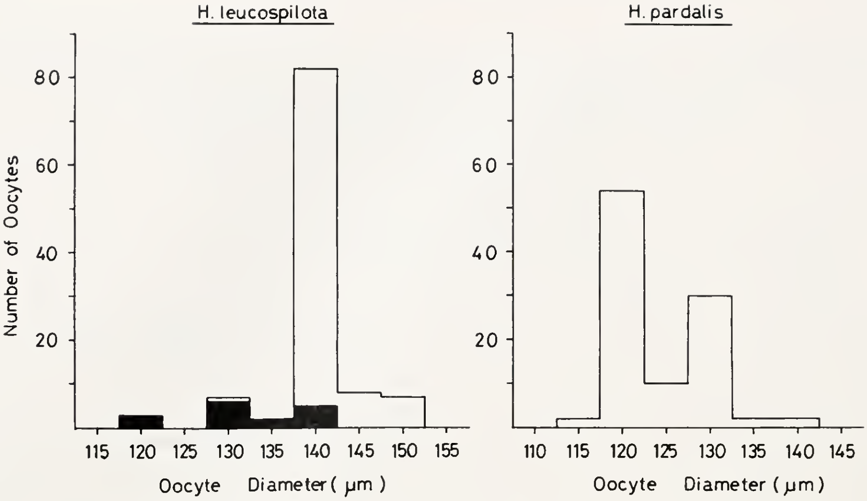

FIGURE 1. Diameter distribution of mature and immature oocytes after DTT treatment (1×10^{-3} M). Open and shaded columns indicate mature and immature oocytes, respectively. Average diameter of mature oocytes: in *Holothuria leucospilota* 141 ± 4 μm (means ± s.d., n = 100) and in *Holothuria pardalis* 124 ± 5 μm (means ± s.d., n = 100). In this sample of *H. pardalis*, all oocytes were induced to mature.

In the case of *H. leucospilota,* however, germinal-vesicle breakdown was not observed in small oocytes (less than 130 μm in diameter), which suggests that these oocytes are not full-grown. Only those oocytes exceeding 130 μm in diameter were studied, on the assumption that these alone are full-grown. Small oocytes (under 130 μm) were not scored.

In *H. pardalis,* only oocytes with a diameter of more than 115 μm were regarded as full-grown.

The diameter distributions of the oocytes are shown in Figure 1.

DTT treatment necessary for germinal-vesicle breakdown

A dose-response curve of oocyte maturation in *H. leucospilota* with various concentrations of DTT solution (1×10^{-5}–1×10^{-1} M) is shown in Figure 2A. Almost 100% of oocytes matured in 5×10^{-4}–1×10^{-2} M DTT solution. At concentrations beyond this range, oocytes rarely matured.

The duration of DTT treatment (1×10^{-3} M) required for induction of oocyte maturation in *H. leucospilota* was determined (Fig. 2D). The curve of germinal-vesicle breakdown *versus* the period of treatment shows that a 10-min treatment induces such breakdown in almost all oocytes and that a threshold exists at 3–5 min in *H. leucospilota* and at 10 min in *H. pardalis* (not shown), at 28–29° C. Nearly all oocytes induced to mature by DTT developed when inseminated (see later sections).

The effects of other disulfide reducing agents and 1-MA were also tested. BAL (5×10^{-3} M) and L-cysteine (5×10^{-2}–1×10^{-1} M) successfully induced 100% of oocyte maturation (Figs. 2B and 2C), although a very high concentration was required in the case of L-cysteine. The process and time course of maturation by these agents were almost the same as those by DTT.

1-MA (1×10^{-6}–1×10^{-4} M) and glutathione (reduced form, 1×10^{-6}–3×10^{-1} M) failed to induce oocyte maturation in *H. leucospilota.*

From these observations, a 10-min treatment with 1×10^{-3} M DTT solution was chosen as the standard procedure for maturation. The treated oocytes were washed with sea water three times to remove DTT and then used as materials in the following experiments.

Process of oocyte maturation

Since holothurian oocytes are spawned at the metaphase of the first meiotic division under natural conditions (Ohshima, 1925a, b; Costello *et al.,* 1957), the earlier processes of maturation have been very difficult to study. Here the process of germinal-vesicle breakdown and meiotic division will be described in some detail, mainly as it occurs in oocytes of *H. leucospilota.*

A large germinal vesicle is situated in the center of each oocyte, and one large nucleolus is observed within it (Fig. 3A). From the oocyte surface numerous radial fibers extend through a transparent jelly layer to a follicle coat (Figs. 3A-3H). A single transparent cytoplasmic process (termed the micropyle process) (Ohshima, 1925a, b; Lindahl, 1932) extends from the oocyte surface through the jelly layer and its tip attaches to the follicle coat (Fig. 3A). Isolated oocytes do not always possess follicle coats; some have been already stripped off (Fig. 3B).

About 10–15 min after the start of DTT treatment, oocytes lose follicle coats, which form clumps near the micropyle process (Fig. 3B) or sometimes near the antipole of the process. In those oocytes which have been stripped of follicle coats, the tip of the process becomes rounded (Fig. 3C). The shape of the process is maintained until the first meiotic division. Jelly coat (20 μm thick) is not removed by DTT treatment.

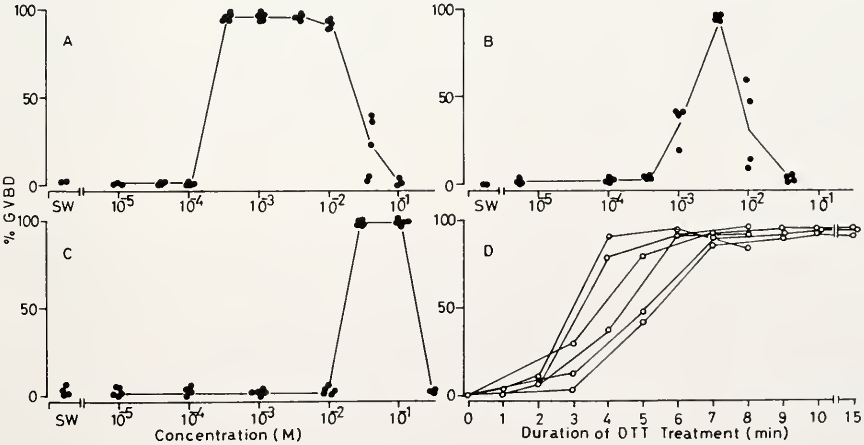

FIGURE 2. Effects of concentrations of DTT (A), BAL (B) and L-cysteine (C) and durations of DTT treatment (1×10^{-3} M) (D) on oocyte maturation in *Holothuria leucospilota.* Each point represents the reponse of 100–180 oocytes. Parts A, B and C show the rates of matured oocytes counted 90–120 mm after the start of treatment with these agents. SW indicates the control oocytes in sea water. In part D, oocytes treated with DTT for various durations were washed with sea water three times and placed into sea water. The rates of germinal-vesicle breakdown (GVBD) were counted 30–50 min after the start of DTT treatment.

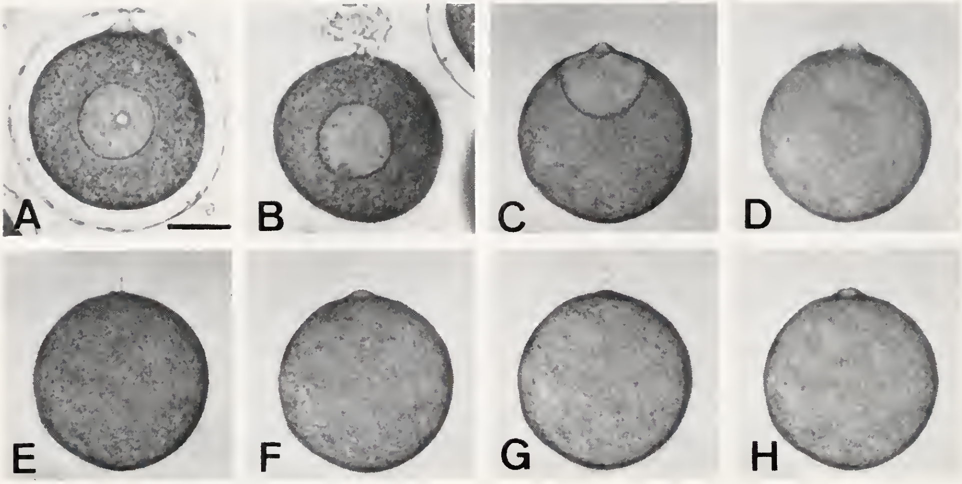

FIGURE 3. Process of oocyte maturation induced by DTT treatment (*Holothuria leucospilota*); (A) and (B) Isolated oocytes, (C) 19 min after the start of DTT treatment, (D) 24 min, (E) 55 min, (F) 65 min, (G) 80 min, (H) 100 min. All pictures are at the same magnification (bar: 50 μm).

At 14 min after the start of DTT treatment, the germinal vesicle begins moving to the site of the micropyle process and at 18 min, assumes the shape of a compressed hemispheroid with one side apparently attached to the inner surface of the oocyte membrane at the site of the micropyle process (Fig. 3C). At 20 min, the nuclear envelope suddenly disintegrates (Fig. 3D). Formation of the first polar body (at 55–60 min) is initiated by contraction of the surface near the micropyle process and subsequent conspicuous elongation of the process (Fig. 3E) and is completed with a pinching-off near its base. Soon after this, a small bulge is formed at the site of the micropyle process (Fig. 3F). At 70–80 min, the second polar body is pinched off from the same place (Figs. 3G and 3H) in the same fashion as the first polar body. The maturation process of *H. pardalis* is almost the same as that of *H. leucospilota*.

Fertilization and the first cleavage

The first discernible reaction of oocytes after insemination is formation of the fertilization cone and wrinkling of the oocyte surface. A few minutes later, the fertilization membrane is formed, although its height is low (Fig. 5A). Immature oocytes, when inseminated, protrude many cytoplasmic processes (probably fertilization cones), which suggests polyspermy. They do not mature or develop.

The spatial relationship between the polar bodies and the fertilization membrane is diverse: insemination before the first meiotic division is followed by formation of both the first and second polar bodies on the oocyte surface within the perivitelline space. When fertilized between the first and second meiotic divisions, oocytes elevate the first polar body with the fertilization membrane and form the second one on the oocyte surface within the perivitelline space. Fertilization after the second meiotic division results in elevation of both polar bodies with the fertilization membrane.

Mature oocytes were inseminated at various stages of the maturation process in order to determine the timing of fertilization appropriate for further development (Fig. 4A). Monospermic fertilization was possible after germinal-vesicle breakdown (20 min). When inseminated thereafter, until 60 min after the start

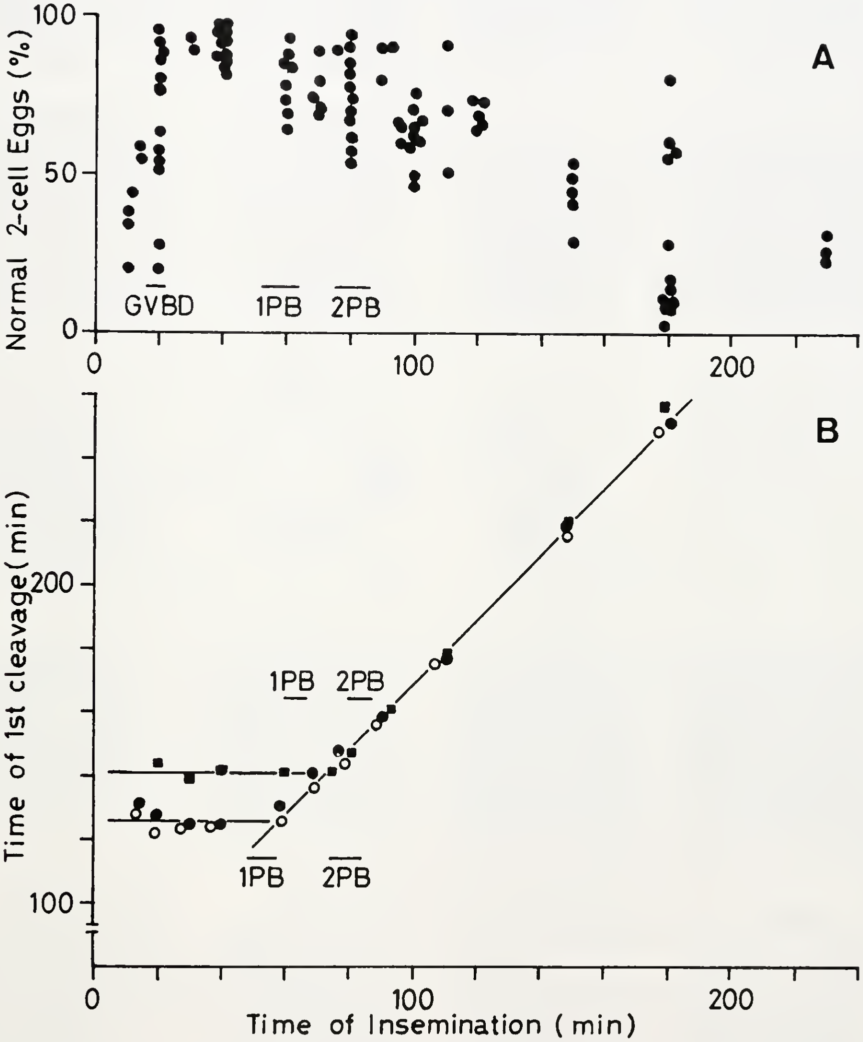

FIGURE 4. Correlations between the time of insemination and the frequency of normally dividing eggs (A), and between the time of insemination and initiation of the first cleavage (B) (*Holothuria leucospilota*). The time of insemination and initiation of the first cleavage was clocked from the start of DTT treatment. The frequency of the dividing eggs was counted with 5-min intervals, and the time when 50% of the eggs divided was referred to as the time of the first cleavage. 1PB and 2PB show the first and the second polar-body formation respectively. In part B, upper signs (1PB and 2PB) are for one experiment from one batch (shaded boxes) and lower signs (1PB and 2PB) are for two experiments from the other batch (open circles and closed circles). The right portion of the curves is the linear regression curve to the plots after the time of formation of the first polar body in these three experiments ($Y = 1.0X + 64.0$, $r = 1.00$). Each point (in A and B) represents the response of 70–230 eggs.

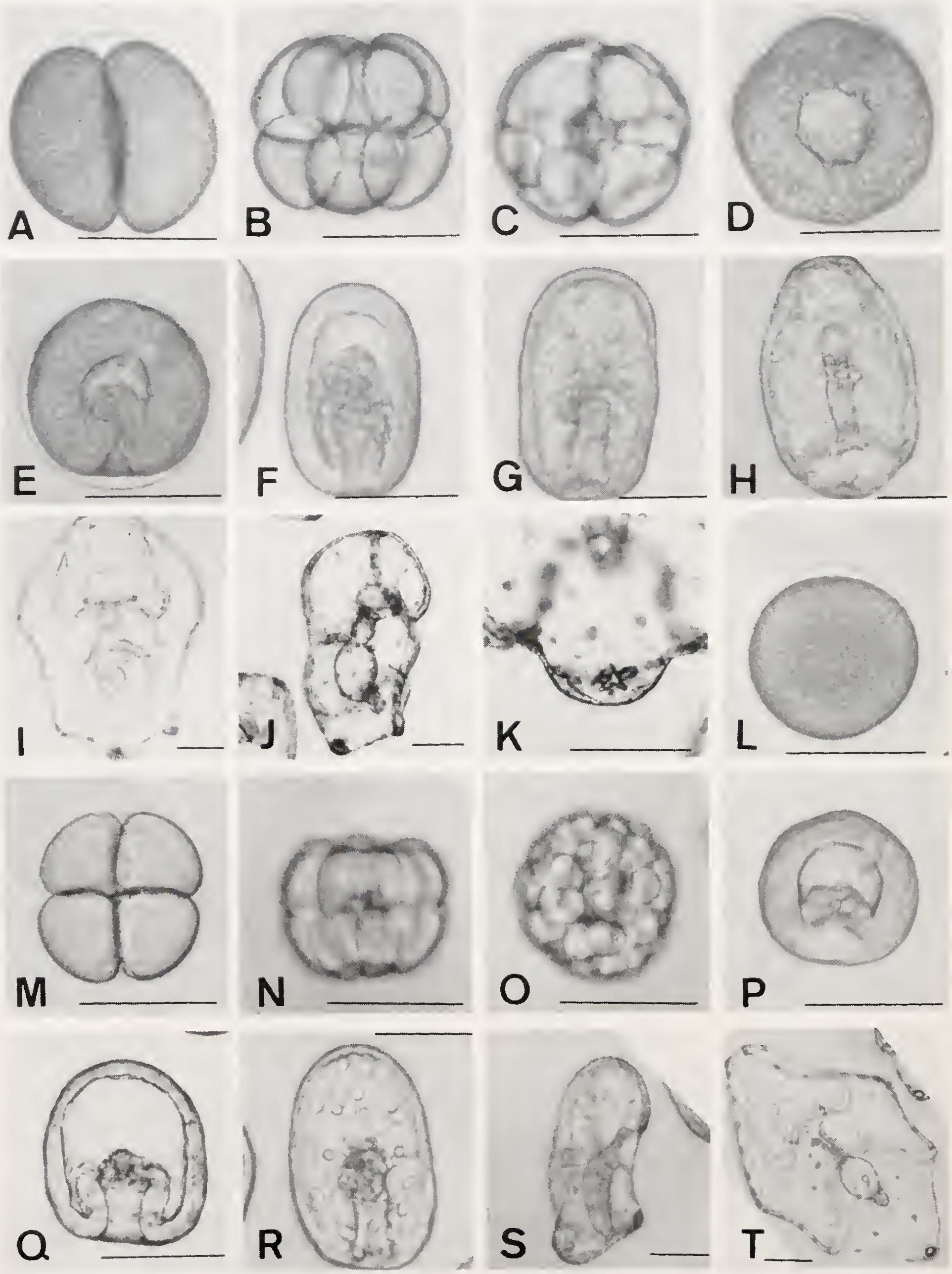

FIGURE 5. Early development of *Holothuria leucospilota* (A-K) and *Holothuria pardalis* (L-T); (A) 2-cell egg (1 hr 30 min after insemination), (B) 8-cell egg (2 hr 40 min), (C) 16-cell egg (3 hr 25 min), (D) Blastula (10 hr 40 min), (E) Early gastrula (13 hr) with invaginating archenteron (just before hatching), (F) Middle gastrula (19 hr), (G) Late gastrula (27 hr) with migrating mesenchymal cells, (H) Early auricularia (39 hr, ventral view), (I) Auricularia (140 hr, ventral view), (J) Auricularia (74 hr, side view), (K) The rear end of 70 hr auricularia, (L) 1-cell egg with two polar bodies, (M) 4-cell egg, (N)

of DTT treatment, 90–100% of mature oocytes divided normally at the first cleavage (later, almost all these oocytes developed to gastrulae). The frequency of normally dividing eggs decreased as insemination was delayed further: The frequency was 30% when they were inseminated at 230 min. The suitable timing of insemination would be, therefore, the period between the completion of germinal-vesicle breakdown and meiotic division.

The interval between insemination and first cleavage was found to depend on the state of maturation of the egg (Fig. 4B). The time of the first cleavage is represented by the time when 50% of the eggs had divided. When oocytes were inseminated at various times before first polar-body formation, they began the first cleavage at the same moment within the same batch (approximately 120–140 min after DTT treatment). Inseminated oocytes formed both polar bodies almost simultaneously with unfertilized mature oocytes. When oocytes were inseminated after the first meiotic division, the interval between DTT treatment and cleavage increased. However, the slope of the linear regression curve is 1.0 (correlation coefficient: $r = 1.00$), which means that the interval from insemination to the first cleavage is constant (about 64 min), and presumably dependent upon the time of fertilization. These results indicate that 1) the process of maturation including egg fertilizability is set prior to and independently of fertilization, 2) the process for the first cleavage could be initiated after the first meiotic division, and 3) after that division, fertilization immediately triggers the processes that result in cleavage.

Development of fertilized eggs

Mature eggs of *H. leucospilota*, inseminated before the first meiotic division (40 min after the start of DTT treatment) were used for observation of further development. At 90 min after fertilization, the first cleavage furrow passed through the animal-vegetal axis (Fig. 5A). About 35 min later, the second cleavage followed the first one with the cleavage plane perpendicular to that of the first division. The third (Fig. 5B) and the fourth cleavages, planes of which were equatorial and meridional respectively, gave rise ultimately to 16 equal blastomeres (Fig. 5C). Up to the 7th cleavage each blastomere divided every 35–45 min, rather synchronously. After the 7th cleavage the divisions became asynchronous and the intervals were prolonged. The surface of the blastula was not wrinkled (Fig. 5D). About 12 hr after fertilization the embryos began to spin within the fertilization membrane. At almost the same time, invagination at the vegetal pole was also initiated (Fig. 5E). The direction of spin of embryos within the fertilization membrane was either clockwise or counter-clockwise as viewed from the vegetal pole. Embryos hatched from the fertilization membrane 13 hr after fertilization. After hatching, all embryos spun counter-clockwise around the animal-vegetal axis as viewed from the vegetal pole, and swam with the animal pole leading, in the same fashion as sea-urchin embryos (Maruyama, unpublished). As gastrulation proceeded, mesenchymal cells began migrating from the archenteron tip into the blastocoel (Fig. 5G). At about 27 hr, the stomodeum at the ventral surface was attached by the bent tip of the archenteron, and cells at

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16-cell egg, (O) 64-cell egg, (P) Early gastrula (13 hr) with invaginating archenteron (just before hatching), (Q) Middle gastrula (14 hr), (R) Late gastrula (23 hr) with migrating mesenchymal cells, (S) Early auricularia (39 hr, side view) with the ciliary ridge and the pore canal, (T) Auricularia (93 hr). Bars indicate 100 μm.

the tip attached to the median dorsal gastrula wall and formed the dorsal pore. At 39 hr, the larvae were at the early auricularia stage (Fig. 5H). The ciliary ridges of the larvae became clear. The position of the anus shifted to the ventral side. At the rear of the body a small birefringent granule appeared. This is probably a calcareous structure, because the birefringence was so strong as to be detectable by a pair of sheets of plastic polaroid. The position of the dorsal pore shifted slightly to the left side as viewed from the dorsal surface (Fig. 5H). At about 46 hr, the ciliary ridge became more and more clear. In side view, a tubular pore canal connected the dorsal pore with the coelom at the position of the oesophagus. At 70 hr, the larvae developed to the typical auricularia (Figs. 5I and 5J). The conspicuous ciliary ridge, which consisted of a single continuous loop, was formed (Figs. 5I and 5J). The birefringent body was observed only in the center of the rear of the larva and now grew to a characteristic four- or five-armed shape (Fig. 5K). The gut was divided into three parts: oesophagus, stomach and intestine. Rhythmic contraction and relaxation of the stomach began. These larvae swam with the head forward and spun counter-clockwise around the long axis of the larval body, as viewed from the rear of the larva.

Development of *H. pardalis* (Figs. 5L-5T) was similar to that of *H. leucospilota,* except that the mature birefringent structure was globular in shape (Fig. 5T).

Discussion

Artificial maturation of full-grown oocytes with BAL, DTT and L-cysteine seems to be, at present, the only useful method for easily obtaining mature eggs of sea cucumbers. The standard procedure presented in this paper was also applicable to immature oocytes of *Holothuria pervicax* and *Holothuria moebi* (Maruyama, unpublished). Oocytes thus matured could be fertilized and developed to the auricularia stage, with the developmental process very similar to that in other species of sea cucumbers reported previously (MacBride, 1914; Korschelt, 1936; Hyman, 1955; Inaba, 1957; Oguro, 1974). This indicates that the eggs obtained in this way are essentially normal.

The cleavage pattern was total-equal, as in the well-known case of cleavage of *Synapta digitata* described by Selenka (MacBride, 1914). About 2 days after fertilization, the ciliary ridge was formed as a single continuous loop, which characterizes the auricularia larva of the sea cucumber (MacBride, 1914; Korschelt, 1936; Hyman, 1955; Inaba, 1957; Oguro, 1974). The birefringent calcareous body, the shape of which was species-specific in each of the two species studied, was formed at the rear of the larval body. Müller and Mortensen described this body as the larval spicule in auricularia larvae of *Holothuria nigra, Holothuria tubulosa* and others whose adult forms are unknown (Mortensen, 1913; Ohshima, 1911).

1-MA (known as the oocyte maturation hormone in starfishes; Kanatani *et al.,* 1969) failed to induce oocyte maturation of sea cucumbers. Identification of a natural maturation hormone specific to the sea cucumber is awaited. At any rate, maturation of oocytes with disulfide reducing agents in sea cucumbers and starfishes indicates that the biochemical mechanisms underlying their processes of maturation probably are similar: The reduction of disulfide bonds may play an important role in the oocyte maturation of both (Kishimoto and Kanatani, 1973; Kishimoto *et al.,* 1976). In the DTT-matured eggs, fertilization-membrane elevation was low. DTT has been believed to have some action destructive to the vitelline envelope, as has been reported in sea urchins (Epel *et al.,* 1970; Vacquier *et al.,* 1972), starfishes (Kishimoto and Kanatani, 1973; Kishmoto *et al.,* 1976) and sea lilies (Holland, 1976).

One significant feature in holothurian oocytes is the existence of the micropyle process, which has attracted attention because it might have a causal relationship with the site of polar body extrusion (Ohshima, 1925a, b). Results reported in this paper indicate that 1) the germinal vesicle migrates to the micropyle process and disappears there, 2) the first and second polar bodies are extruded from the process and, 3) the first cleavage furrow passes near the site of polar bodies. These observations favor the hypothesis that the micropyle process is a preformed structure, laid down during oogenesis, which participates in meiosis and determination of egg polarity. Thus, the micropyle process might itself manifest the "animal pole."

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SUMMARY

Isolated oocytes of the sea cucumbers *Holothuria leucospilota* and *Holothuria pardalis* were induced to mature with 2,3-dimercapto-1-propanol (BAL), dithiothreitol (DTT) and L-cysteine. 1-Methyladenine, known as the maturation-inducing hormone of starfishes, failed to induce oocyte maturation of the sea cucumbers. Observation of the maturation process revealed that the germinal-vesicle of the oocyte treated with DTT migrated to the micropyle process and broke down there, where polar bodies were subsequently formed. After insemination, mature eggs obtained with DTT treatment developed to the typical auricularia larvae.

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SYMBIOTIC ALGAE AND ^{14}C INCORPORATION IN THE FRESHWATER CLAM, *ANODONTA*

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Freshwater clams of the genus *Anodonta* have been reported to associate with zoochlorellae which are the symbiotic counterparts of the free-living *Chlorella* and which inhabit a variety of freshwater invertebrates. The first extensive discussion of the clam-algae association was by Goetsh and Scheuring (1926), who described the zoochlorellae as being distributed throughout the inner tissue layers of the posterior mantle, the posterior region of the gills, and the posterior foot. In other organisms, especially the green hydra, *Hydra viridis,* and *Paramecium bursaria,* zoochlorellae have been clearly shown to augment the host's growth (Muscatine and Lenhoff, 1965; Karakashian, 1963) via products produced by the algae and released to the host's tissues (reviewed in Smith *et al.*, 1969). In the present work the zoochlorellae associated with *Anodonta* are analyzed for their ability to photosynthesize and translocate products to the host *in vivo* and to the medium *in vitro*. The fate of translocated products in the host is examined. Data are presented regarding the degree of algal infection among individual clams of different size. The distribution of symbionts among the various infected tissues of individuals is also assessed.

Materials and Methods

Experimental organisms

Specimens of *Anodonta* (tentatively identified as *Anodonta grandis*) were collected during the months of July and August from Windy Pond, a freshwater reservoir located about 10 miles north of Woods Hole, Massachusetts. Following collection the animals were maintained at room temperature under ambient light in aerated tap water until used in experiments. Animals were also collected for the purpose of determining their wet weight and degree of algal infection. These determinations were done in the field and the animals returned to their habitat after analysis.

Algal symbionts were harvested from green specimens of *Anodonta* by homogenizing pre-dissected clam tissues in millepore filtered (0.4 μ) pond water (MFP) using a Waring blendor. The crude homogenate was filtered through five layers of cheesecloth and the resulting filtrate was further homogenized using a Dounce homogenizer. The algal cells were sedimented from this homogenate by low speed centrifugation ($500 \times g$) using a clinical centrifuge. The algal pellet was washed 3–5 times with MFP and finally resuspended in MFP for use in labelling experiments.

Incubation with $^{14}CO_2$

Whole animals were incubated for 3 hr in 300 ml of MFP containing 50 μCi $NaH^{14}CO_3$. During the incubation the animals were illuminated by a bank of six

15-watt fluorescent tubes which emitted 20 microeinsteins of photosynthetically active radiation (PAR) as measured by a Licor quantum sensor and counter. In dark experiments the animals were incubated in containers wrapped with aluminum foil.

Isolated algae (0.5 ml wet-packed) were incubated in 10 ml of MFP containing 10 μCi $NaH^{14}CO_3$. In some instances isolated algae were incubated in MFP buffered at pH7, 6.4, or 5.5, with McIlvane's citrate buffer. Algal suspensions were either illuminated by a bank of six 15-watt fluorescent tubes (20 microeinsteins PAR) or kept in the dark by wrapping the incubation vessels with aluminum foil. Incubations were carried out for $2\frac{1}{2}$ hr at room temperature after which the symbionts were centrifuged from the medium. Acidified samples of algae and medium were analyzed for ^{14}C by suspension in Aquasol and counting in a Beckman L.S. 100 scintillation spectrometer. Counting data were corrected for background and quench (quench never exceeded 10%).

Distribution of radioactivity in clam tissue

Following incubation in radioactive medium, animals were rinsed several times with MFP and dissected. Gills, mantle tissue, foot, and digestive gland were removed and homogenized separately in MFP using a Potter-Elvenhejem tissue homogenizer. The homogenates were fractionated after the method of Roberts *et al.* (1957) yielding a trichloroacetic acid (TCA) soluble fraction (mainly small molecules), an alcohol-soluble fraction (mainly lipids), a hot-TCA-soluble fraction (nucleic acids) and an alcohol- and TCA-insoluble fraction (proteins). In one experiment, blood collected by heart puncture was also fractionated. Samples of chemical fractions were transferred to scintillation vials containing Aquasol and counted in a Beckman L.S. 100 scintillation spectrometer. Counting data were corrected for background and sample quench.

Protein and chlorophyll determinations

The amounts of chlorophyll and protein contained in various clam tissues were determined by analyzing tissue discs. These discs were cut using a 1 cm cork borer and were homogenized in a tissue grinder following premincing with a razor blade. Algal cells were centrifuged from these homogenates, washed twice with MFP and finally extracted with absolute methanol for 24 hr at 5° C. Extracts were analyzed for total chlorophyll at 650 and 665 mμ by the method of MacKinney (1941) using a Bausch and Lomb Spectronic 20. The animal tissue homogenates were anlyzed for protein content by the Lowry *et al.* (1951) method using a standard curve prepared from partially purified *Anodonta* protein.

RESULTS

Specimens of *Anodonta* were found at depths ranging from about 0.5–2 M. The clams lay superficially embedded in soft mud with the posterior margins of their valves just protruding above a fine layer of silt.

Not all clams exhibited an algal infection. As judged by superficial visual inspection, the degree of infection appeared correlated with the size of the clam: large clams ($\bar{x} = 72.03$ g ± s.d. 13.46, n = 14) exhibited heavy infection, especially of the gills and mantle; small specimens ($\bar{x} = 40.20$ g ± s.d. 19.70, n = 16) exhibited no external evidence of infection. The larger specimens exhibited valves

TABLE I

Amount of chlorophyll and protein in 1-cm tissue discs taken from various regions of Anodonta *specimens. Data expressed as* $\bar{x} \pm$ *s.d.,* $n = 3$ *discs for each determination.*

	Chlorophyll (μg)	Protein ($\mu g \times 10^{-3}$)	Chlorophyll/Protein $\times 10^{-4}$
posterior gill	1.67±0.37	5.22±1.20	3.2
mid gill	1.13±1.20	4.58±2.50	2.5
anterior gill	1.10±1.50	3.97±1.25	2.8
mid mantle	6.53±2.95	6.98±2.45	9.4
posterior mantle	0.52±0.49	1.73±1.19	3.0

that were pocked, abraded, and on occasion completely perforated, thus exposing the underlying mantle.

When clams were dissected it was observed that the green symbionts were more densely aggregated towards the posterior end of the animal. The folded, leaf-like organization of the mantle and gills lent itself to the preparation of tissue discs cut from these organs. Analysis of these discs for chlorophyll (originating mainly from zoochlorellae) gave an indirect estimate of the relative distribution of symbionts within the gills and mantle. Table I lists the chlorophyll content, protein content, and chlorophyll : protein ratio of discs cut from the gills and mantle of large (60–80 g) clams. From Table I it can be seen that the greatest amount of chlorophyll and by inference the greatest number of symbionts were detected in the mid-mantle region. This region was also the greenest. The lowest cholorphyll per disc was measured in the posterior mantle (Table I). This region of the mantle appeared darkly pigmented, obscuring the symbionts and making it impossible to judge the degree of infection by eye. The chlorophyll : protein ratio was fairly constant among the discs from the various regions ($\approx$ 0.0003, Table I) with the exception of the mid-mantle region, which exhibited a ratio of 0.00094 (Table I), reflecting the greater amount of chlorophyll in this region.

The data in Table II show that the total incorporation of ^{14}C by animals incubated in the light (average of experiments I and II) is approximately seven times the total incorporated by animals in the dark (average of experiments III and IV). Further, Table III shows the distribution of ^{14}C among the various chemical fractions prepared from tissues of clams incubated in either light or dark. The largest amount of ^{14}C incorporated occurs in the TCA-soluble fractions from clams incubated in the light. Among the tissues analyzed, the gills and blood showed the greatest amount of ^{14}C in this fraction. This fraction also contained the highest percentage of ^{14}C (75–95%) of all fractions irrespective of the tissue origin. By contrast, the TCA-soluble fractions from tissues of dark-incubated clams contained approximaely $\frac{1}{10}$ to $\frac{1}{5}$ as much ^{14}C as their illuminated counterparts. The distribution of the label in these fractions differed strikingly from that observed in the light experiments. Whereas the TCA-soluble fraction invariably exhibited the highest percentages of ^{14}C (75–95%) in illuminated clams, it contained lower percentages (27–67%) in the dark-incubated clams. There was a concomitant increase in the percentages of ^{14}C in the alcohol-soluble and hot-TCA-soluble fractions from dark-incubated clams, amounting to some 10–40%. By comparison, with the exception of the mantle tissue of experiment I, the per cent of ^{14}C in the alcohol-soluble and hot-TCA-soluble fractions of experiments I and II never exceeded 9%. Finally it can be seen from Table II that the total ^{14}C measured

Table II

Distribution of radioactivity in different fractions of clam tissue in four experiments. For each entry the first figure is the percent ^{14}C in that fraction; the second figure is the total ^{14}C expressed as dpm/mg clam protein in the initial tissue homogenate.

	TCA sol.	Alc. sol.	Hot TCA sol.	Alc. insol., Hot TCA insol.
Incubated in Light				
Experiment I				
Digestive gland	87.6, 3,490	5.6, 222	4.8, 192	2.2, 86
Foot	91.3, 2,453	1.1, 29	4.6, 124	3.0, 81
Gills	82.3, 9,529	4.1, 477	8.3, 961	5.3, 611
Mantle	75.7, 2,882	4.8, 184	17.8, 654	2.3, 86
Total (all fractions and tissues) = 22,061 dpm/mg protein				
Experiment II				
Digestive gland	89.3, 1,072	4.2, 51	3.6, 44	2.8, 34
Foot	88.2, 2,369	6.4, 172	2.3, 64	2.9, 80
Gills	79.1, 3,658	7.3, 339	7.5, 349	6.0, 278
Mantle	82.8, 3,261	6.8, 269	7.7, 305	2.6, 101
Blood	95.4, 20,384	2.5, 536	1.4, 300	0.6, 138
Total (all fractions and tissues) = 33,804 dpm/mg protein				
Incubated in Dark				
Experiment III				
Digestive gland	27.2, 490	20.8, 375	43.9, 792	8.1, 146
Foot	41.7, 220	29.8, 157	21.6, 114	6.8, 36
Gills	60.9, 331	11.0, 56	22.9, 117	5.3, 27
Mantle	32.2, 306	10.3, 123	39.5, 473	18.0, 216
Total (all fractions and tissues) = 3,959 dpm/mg protein				
Experiment IV				
Digestive gland	48.4, 531	12.8, 141	35.4, 390	3.3, 36
Foot	37.1, 117	33.9, 107	24.8, 78	4.1, 13
Gills	54.8, 201	16.2, 60	21.8, 80	7.1, 26
Mantle	66.9, 269	14.7, 59	15.7, 63	3.7, 11
Blood	44.7, 678	41.8, 634	6.5, 98	7.1, 108
Total (all fractions and tissues) = 3,700 dpm/mg protein				

in the alcohol-soluble, hot-TCA-soluble, and alcohol-insoluble, hot-TCA-insoluble fractions ranged from 11 dpms to 961 dpms in the light experiments and 27 dpms to 490 dpms in the dark experiment. In all experiments the alcohol-insoluble, hot-TCA-insoluble fractions contained the lowest activity and the lowest percentage of the ^{14}C incorporated.

Table III

Effect of pH on translocation of ^{14}C in vitro *by symbionts harvested from* Anodonta *specimens*

pH	Total ^{14}C fixed (dpm)	Percent of ^{14}C in medium
7.4	1,939,655	10.8
6.5	1,074,935	16.6
5.5	828,455	19.6

Table III shows the results of *in vitro* experiments exposing isolated symbionts to $NaH^{14}CO_3$ in MFP adjusted to three different pH's. Analysis of acidified samples showed a significant percentage of acid-precipitable ^{14}C in the medium at pH 7.4. This percentage nearly doubled at pH 5.5. Total ^{14}C fixed (symbionts + medium) decreased with decreasing pH.

Discussion

The degree of algal infection of individual clams appears correlated with the size/age of the clams. Large (older) clams exhibit the greatest degree of infection. While the details of infection are not known, the data suggest that infection may be a progressive process beginning, perhaps, in the clam's early adulthood. Individual clams might acquire symbionts from the environment and these could multiply slowly within the host's tissues. Alternatively, infection with zoochlorellae could be continuous, with symbionts constantly being derived from the environment and added to the standing crop in the clam.

Valves of the largest clams were often chipped and pitted, giving rise to a thinner and more translucent shell material in the central portion of the valve. On occasion there were intense green areas on the tissues underlying these "windows." Examination of tissue from these regions revealed a high density of zoochlorellae. These observations suggest that zoochlorellae tend to congregate where there is more light, perhaps as a result of enhanced cell division. Goetch and Scheuring (1926) attributed this pitting and chipping of the valves to effects of the parasitic [sic] algae. Examination of valves from specimens of *Anodonta* collected at Windy Pond revealed that the pitting took place inward from the outer surface, suggesting some external environmental cause of degradation of the shell material.

The major symbiont-bearing tissues of the clams were the gills and mantle. These were fairly uniform with respect to the distribution of chlorophyll (Table I) with the exception of the mid-mantle. This region exhibited over three times the chlorophyll per unit protein of any other region sampled. This part of the mantle, which invariably lay under the chipped and pitted portion of the clam's valves, looked greener than other parts. Occasionally aggregations of symbionts were observed in the superficial tissue of the foot.

Data from four experiments, in Table II, strongly suggest the viability and photosynthetic capacity of the zoochlorellae associated with *Anodonta* tissue. Incorporation of ^{14}C by clams illuminated in the light averages seven times greater than dark incorporation. Fixation of ^{14}C by clam tissues is not insignificant. However, in terms of respiratory biomass, the clam tissue undoubtedly exceeds the zoochlorellae by several orders of magnitude. Hence the apparent large dark-fixation component of the total ^{14}C fixed. By comparison, light fixation of ^{14}C in hydra is ten times greater than dark incorporation (R. Pardy, unpublished data), and the respiratory biomass of the host in the hydra association is less than in the clam.

Radioactivity from ^{14}C is found in all chemical fractions of the host (Table II) with the largest proportion occurring in the small-molecule fractions of tissues from illuminated clams. The appearance of ^{14}C in the nucleic acid and protein fractions indicates that some ^{14}C, probably as small-molecule precursors, is metabolized into macromolecules. By comparison, ^{14}C from fractions of tissues from dark-incubated clams is generally more evenly distributed among the various chemical fractions, with the exception of the alcohol-insoluble, hot-TCA-insoluble fraction, which generally contained less. The digestive gland (experiments III and IV) and mantle (experiment III) showed a comparatively high percentage of ^{14}C in the nucleic acid fraction. The reason for this is not clear: either these tissues metabolize the products of dark fixation differently or the results reflect experimental variation in the organisms or analytical techniques.

The ultimate fate of the large fraction of TCA-soluble molecules in the illuminated clams is only suggested by the data in Table II. Clearly some may be metabolized into lipids and macromolecules. However, the activity of these fractions is not appreciably higher than that observed in the corresponding fractions from the dark-incubated animals. Either the small molecules are rapidly respired or the duration of the experiments was too short to observe significant metabolism of these molecules into the other biochemical components. In the green hydra, Roffman & Lenhoff (1969) showed progressive movement of ^{14}C from the TCA-soluble fraction to the other fractions over several hours. Long-term experiments on *Anodonta* were not performed during the present investigation.

In other symbioses involving zoochlorellae, the source of ^{14}C in the host tissues is translocated metabolites that originate from symbiont photosynthesis (Muscatine, 1965; Muscatine *et al.*, 1967). Invariably, significant amounts of labelled organic material can be found in the medium when these symbionts are incubated *in vitro*. A similar finding has been obtained from *Anodonta* zoochlorellae (Table III). Depending upon the pH of the incubation medium, isolated symbionts contribute up to nearly 20% of their total fixed ^{14}C to the medium as acid-stable (organic) carbon. This argues for the existence of a translocating system that operates when the symbionts are *in situ* as well, providing a basis for photosynthetically fixed ^{14}C to enter the host's metabolic network. Table III also shows that the percentage of ^{14}C in the medium increases with decreasing pH. A similar dependency of translocation on pH has been shown for green-hydra symbionts (Muscatine, 1965; Cernichiari *et al.*, 1969). Hence it seems that the *Anodonta* symbionts may be related, at least functionally, to those zoochlorellae that inhabit hydra. The apparent decrease in total incorporation of ^{14}C with decreasing pH (Table III) might result from either an inhibition of photosynthesis and/or decreased availability of $NaH^{14}CO_3$.

The overall role of zoochlorellae in augmenting the metabolic and respiratory demands of *Anodonta* is not known. The products of translocation have not been identified. Moreover, the cytological and cellular aspects of this symbiosis have not been investigated adequately to define the spatial relationships between host and symbiont. Clearly, further research at both levels is needed before statements regarding the efficacy of the symbiosis and role of the symbionts can be made.

I wish to thank William Fitt, James Batty, and Matt Pardy for help in collecting some of the specimens; and the staff of the Marine Biological Laboratory, where part of the work was done.

SUMMARY

1. Specimens of a freshwater clam *Anodonta* were found to be symbiotized with zoochlorellae. Larger, hence older, clams showed a greater degree of infection with algae.

2. In the light, clams exposed to the precursor $NaH^{14}CO_3$ showed a greater incorporation of ^{14}C than corresponding control animals incubated in the dark.

The difference is thought to result from the photosynthetic activities of the zoochlorellae.

3. Chemical fractionation of selected host tissues revealed that in the light, ^{14}C appeared in greatest amount in the small molecule fraction though it was also detected in other fractions.

4. The major source of ^{14}C in the hosts incubated in light is thought to be photosynthetic products translocated by the zoochlorellae. This view is strengthened by data showing that the symbionts can translocate to the medium when the algae are incubated *in vitro.*

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SOME FACTORS AFFECTING PRESUMED MIGRATORY FLIGHT ACTIVITY OF THE CONVERGENT LADYBEETLE, *HIPPODAMIA CONVERGENS* (COCCINELLIDAE: COLEOPTERA)

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Insect migration is a distinct behavioral, ecological and physiological phenomenon (Dingle, 1972; Kennedy, 1961; Johnson, 1969). Behaviorally, migratory flight can be distinguished from "trivial" or appetitive flight because it is typically prolonged, continuous, undistracted flight which is not arrested by stimuli which would elicit settling behavior during appetitive flight, such as food, an oviposition site, or a mate. Response to such "vegetative" stimuli seems to be inhibited during a migratory flight, although the inhibition ultimately diminishes and settling behavior can again be evoked (Kennedy, 1961).

Ecologically, insect migration may be either an escape in space from unfavorable habitats or an "investment" of a portion of a population in colonizing and exploiting resources in a habitat some distance from the one in which adult emergence of the migrants occurred. Migratory behavior, then, is often displayed by only a portion of a population in response to appropriate environmental cues such as photoperiod, temperature, food quality, population density, and moisture. Migrants are often denizens of temporary or early successional habitats, with very high reproductive potential and great capacity for rapid exploitation of a newly invaded habitat (Johnson, 1969; Dingle, 1972).

Physiologically, migration is usually post-teneral and pre-reproductive, at least in females (Dingle, 1972; Johnson, 1969), and oogenesis or the presence of fully developed ovaries has often been shown to inhibit migratory flight in reproductive females (Rankin, 1974, 1978). Migrants, like insects in adult diapause, typically have undeveloped ovaries and hypertrophied fat bodies, and many of the environmental cues which induce adult diapause elicit migratory behavior where it occurs. There seems to be a hormonal component to migratory behavior in that both juvenile hormone (JH) (Rankin, 1974) and the adipokinetic hormone from the corpus cardiacum (Goldsworthy *et al.,* 1972) have been shown to stimulate migratory behavior in some species. Juvenile hormone has also been shown to cause flight muscle degeneration in a few species (Borden and Slater, 1968; Davis, 1975; Rankin, 1978).

Hippodamia convergens is one of the most widespread of the American coccinellids, occurring throughout the western, central and southern United States. It is an economically important predator of aphid and mite pests on cultivated crops and in fact is commercially available as a natural control agent for such pests. It is highly mobile, however—capable of migrating hundreds of miles under some circumstances (Hagen, 1962), a characteristic which often considerably reduces its effectiveness as a natural pest-control agent. This paper examines some of the characteristics of migratory behavior, reproduction, and adult diapause in *H. convergens* as a first step in an investigation of the physiological basis of migration in this species.

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Materials and Methods

Insect sources and maintenance

Beetles used in these experiments were obtained from several sources:

1. For some experiments adult *Hippodamia convergens* (Guérin-Méneville: Coccinellidae) were purchased from the Bio-Control Company, Auburn, California. Animals obtained from this source were kept in an unilluminated refrigerated chamber at 7° C until needed. One day before an experiment, animals were removed from the low-temperature incubator and placed in small plastic containers in large walk-in environmental chambers in either a 16L:8D, or a 12L:12D, 24° C photoperiod regime
2. Some animals, including adults, larvae, and eggs, were collected from the fields and gardens around Austin, Texas. Animals collected from these areas were placed in plastic rearing containers in environmental chambers in either a 16L:8D, or a 12L:12D, 24° C regime.
3. For some experiments animals were collected as adults from their mountaintop aggregation sites in the Davis Mountains in western Texas, particularly from the top of Mt. Locke. Such animals were transported back to Austin in insulated containers and placed in plastic rearing containers in environmental chambers either at 7 or 24° C.
4. Some of the ladybeetles were second-generation lab-reared animals, maintained on either a 16L:8D or a 12L:12D, 24° C environmental regime.

Beetles were fed either frozen potato aphids, *Macrospihon euphorbia,* or various species of live aphids collected around Austin, Texas. Live aphids were given to the beetles on small sprigs of their host plants, which were placed in the plastic rearing containers.

Flight testing procedure

Insects were flight tested using a modified version of the tethered-flight technique of Dingle (1966). Each animal was suspended from its pronotum by a toothpick tipped with melted beeswax and flight was stimulated by moving the animal rapidly through the air in a figure-8 motion five times, or until it began to fly if it started to fly before it was given 5 trials. Flying animals were then placed in front of a low-speed fan and flight activity was timed. All flight tests were performed between 5½ and 9½ hr after lights on because there appears to be a diel periodicity to flight behavior in this species which peaks during that period of the day (Rankin and Rankin, unpublished observations).

Autopsy procedures (determination of weight of female reproductive tract or testes)

Animals were fixed in Heidenhain's susa (Galigher and Kozloff, 1964) for 48 hr. The fixative was then drawn off and the animals were washed repeatedly with 70% ethanol and refrigerated in this solution at least 48 hr before dissection. The entire fixed female reproductive tract was removed, cuticle was carefully teased from the base and the blotted tract was weighed on a Cahn electrobalance, Model M-10. Testes were removed from fixed males and weighed in the same way.

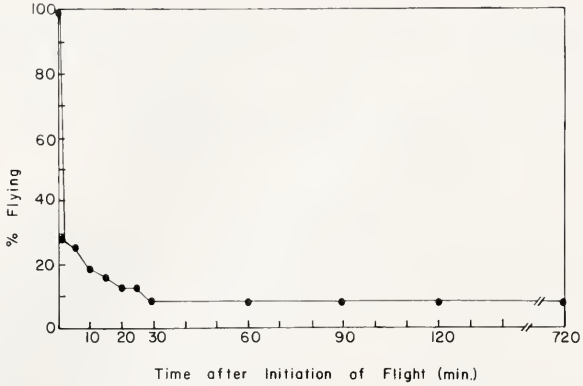

FIGURE 1. Tethered flight activity (up to 12 hr of flight) in a population of *H. convergens* (n = 60). Animals were allowed to fly for 20 hr before they were stopped and removed from the tethers. Those still flying at 12 hr were observed at intervals up to 20 hr and were all flying at each observation. Only flight up to 12 hr was graphed, however, since flight was not monitored continuously after that time.

RESULTS

Tethered flight behavior in laboratory and field populations

Initially we wished to determine whether a tethered flight procedure would provide a useful measurement of migratory behavior in this species. A group of 60 *H. convergens* were flight tested using this technique and the total time up to 20 hr was recorded (beetles were stopped after about 20 hr). As Figure 1 indicates, we observed a sharp drop in flight activity at 1–2 min of flight and a more gradual decline until 15–20 min of flight. Beetles that flew 30 min nearly always continued to fly much longer. The experiment was repeated three times using field-collected animals in December, March, and June. In each case a similar pattern in flight behavior was observed: The timing of short flights was virtually identical to that observed in the first experiment, but the percentage of beetles flying 30 min or more varied at the three times, being 68% in March, 35% in June, and 25% in December. We felt, therefore, that performance of a 30-min flight was a reasonable indication of a migratory response.

In order to determine more directly whether populations of *H. convergens* exhibit a greater tendency to migrate (to display long tethered-flight behavior) at certain times of the year than at others, field-collected beetles were flight tested at intervals throughout the year. Field collections were made from Mt. Locke in the west Texas Davis Mountains and from fields and gardens in and around the University of Texas Brackenridge Field Laboratory in Austin. Animals were flight tested the afternoon of their capture or the following day. Beetles were observed in large aggregations on Mt. Locke from July, 1977, to February, 1978. They began to appear around Austin in late February in 1976 and again in 1977. They were no longer found after June in 1976 and July in 1977.

Newly arrived animals collected around Austin in March, 1977, showed about 50% long-flight activity. During April and May, times of plentiful aphid supplies and oviposition, long-flight activity was depressed to about 20–30%. By late May and June, long-flight behavior had increased to about 60%. Newly aggregated

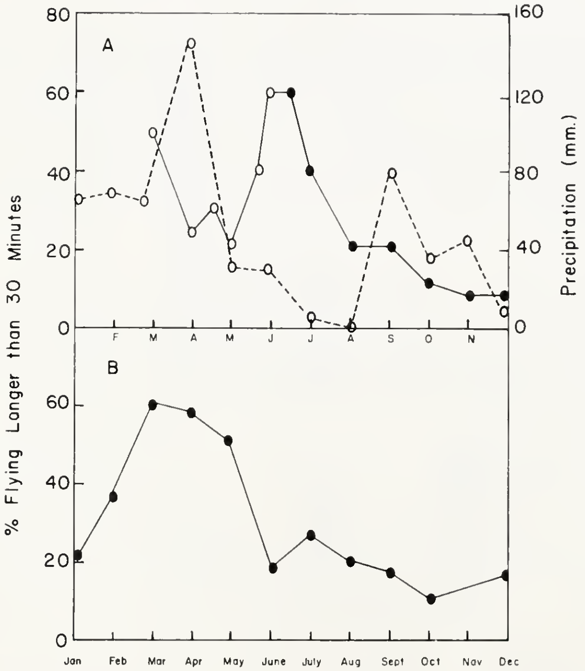

FIGURE 2. A. Flight activity of field collected beetles at monthly intervals from Mar.–Dec., 1977. Open circles, solid line = flight activity of beetles collected in aphid infested fields around Austin, Texas; closed circles, solid line = flight activity of beetles collected on top of Mt. Locke in the Davis Mountains of western Texas; open circles, dashed line = monthly precipitation in the Austin area, Jan.–Dec., 1977. B. Flight activity of California beetles obtained in Nov. and again in June from Bio-Control Co. and held at 7° C until 3 days prior to the flight test. Beetles were tested at monthly intervals, 3 days after being placed in a 16L : 8D, 24° C environmental regime.

beetles collected from Mt. Locke in July and August displayed only a 20% migratory population. The proportion of long flights declined through the last collection, which occurred at Mt. Locke in December (See Fig. 2A).

These data suggest a periodic change in willingness to make long flights within the *H. convergens* population. We were interested in investigating this possibility more directly. California beetles which were obtained from Bio-Control in November, 1978, and maintained at 7° C were periodically removed from the low-temperature incubator, held for 3 days at 24° C, 16L : 8D, and then flight tested. A significant change in the proportion making long flights was observed over the 11-month testing period (Fig. 2B). Not surprisingly, the pattern of change under these highly artificial conditions was not identical to that observed in the field-collected animals. However, a marked increase in flight activity did occur from February to June. The proportion of animals making a long flight was 20% in January, rose to 30% in February, and increased to nearly 60% during March, April, and May. By the June flight tests the proportion of long flights had again diminished to 20%. The beetles flight tested in June were from a new shipment of beetles. These animals had just been collected as new arrivals at their hibernation sites in California by Bio-Control company.

Another behavioral change in the beetles was observed over the storage period from November to May. When beetles were removed from the low-temperature incubator and placed at 24° C in December they would not feed, even when given live aphids, for 4 days. By February they would begin to feed after 2 days at 24° C and by May they would feed on the first day they were placed at the higher temperature. It would seem that there is a change during the diapause period in threshold of response to both migratory and food stimuli.

Effects of adult age, photoperiod, and food quality on flight behavior

Because migration is often a post-teneral, pre-reproductive phenomenon (Johnson, 1969), we investigated the frequency of long flights in young adult *H. convergens.* Beetles used in this experiment were reared from eggs in either 12L:12D or 16L:8D, 24° C environmental regime. They were isolated after hatching because hatchlings eat nearby eggs and other larvae, and were fed on frozen aphids throughout the larval period. Upon adult emergence, adults in each environmental regime were divided into two groups. One group was maintained on frozen aphids (an adequate but sub-optimal food) while the other was provided with fresh aphids. All beetles were flight tested daily from the first day after adult ecdysis. Animals maintained under either short- or long-day conditions with either frozen or live aphids exhibited a short, 1–2-day teneral period (as assessed by superficial observation of cuticle hardness) and little or no flight activity occurred during that time (Figs. 3A and B). Animals maintained under either photoperiod and fed on frozen aphids showed a high incidence of long-flight be-

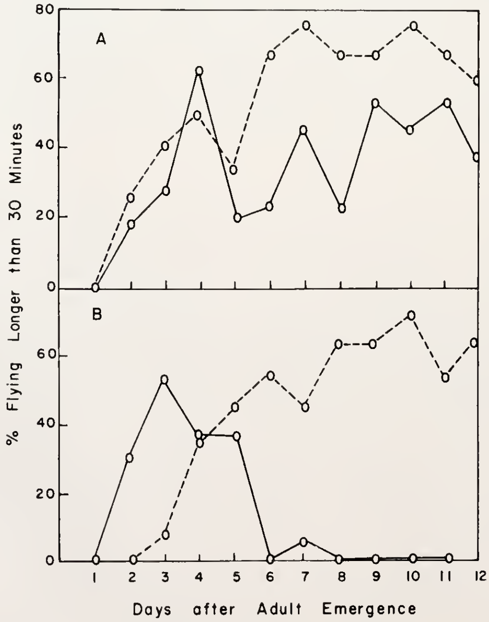

FIGURE 3. Daily flight activity after adult emergence among animals reared under a 12L:12D, 24° C (A) or a 16L:8D, 24° C (B) environmental regime and fed on either frozen aphids (dashed line, n = 11 both A & B) or fresh aphids (solid line, n = 13 both A & B). Differences between frozen and fresh aphid fed groups is significant at the 0.001 level for both A and B.

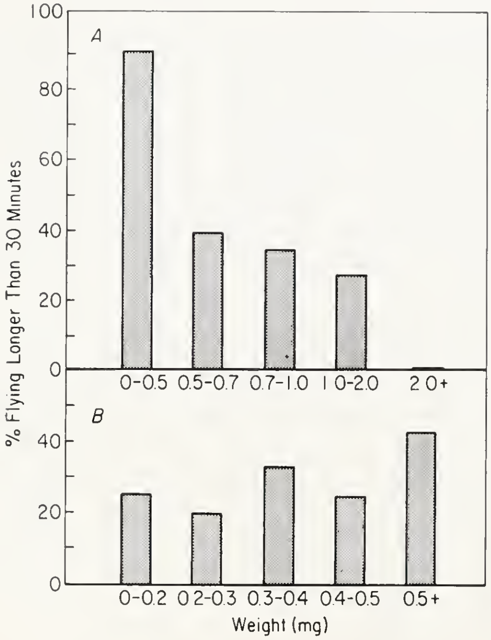

FIGURE 4. Flight activity of female (A) or male (B) *H. convergens* collected in mid-summer (long-day photoperiod) around Austin, Texas with respect to weight of the fixed reproductive tract (females, n = 38) or testes (males, n = 35).

havior by day 4 after emergence. This was maintained throughout the flight-testing period. None of the frozen-aphid-fed females in either photoperiod group displayed oviposition behavior until 21 days after adult emergence.

Animals under either photoperiod regime, and receiving fresh aphids daily, displayed a peak of long flight activity 3–4 days after emergence followed by a decline to much lower levels than that observed in animals maintained on frozen aphids. There appeared to be a photoperiodic response among the live-aphid-fed animals which was not observed in the other groups. Under the long photoperiod regime, long flights were virtually eliminated from the population by 6 days after emergence while under the shorter photoperiod, long flights continued among 20–40% of the population throughout the testing period. Oviposition occurred by day 6 in both groups. Animals fed on live aphid prey were much shorter-lived than those fed on frozen aphids. By 60 days after emergence all of the beetles fed on live aphids were dead while all of the animals fed on frozen aphids that were alive at the end of the testing period (n = 11) were still alive 60 days after emergence.

The relationship between flight activity and reproductive development was examined by flight testing 38 females and 35 males, fixing them in Heidenhain's susa, and weighing the reproductive tract (females) or the testes (males). Beetles were collected from aphid-infested areas around Austin in mid-summer, 1978, and were flight tested immediately after capture. Animals were then fixed and examined to determine reproductive development. Females showed a clear inverse relationship between flight and reproductive development (Fig. 4A) while males showed no correlation between testes weight and flight activity (Fig. 4B).

This experiment was repeated using females collected (as above) in short-day conditions between March 1–15 in Austin, Texas. Flight tests were performed as above (n = 38). The relationship between flight and ovarian development is rather different under short photoperiods. The percent making long flights increases with ovarian weight up to about 1-mg tract weight and then decreases, with some long flight behavior continuing under short photoperiods until the ovary is completely developed (3 mg tract weight) (Fig. 5). The apparent difference in the "oogenesis-flight syndrome" (Johnson, 1969) with photoperiod may have important consequences in the life history strategy of *H. convergens* (see Discussion).

To determine the relationship between reproductive development, photoperiod, and food quality in female *H. convergens,* four groups of 30 females each were removed from the 7° C incubator, placed in either a long-day or a short-day regime and given either frozen or live aphids as food. Every day beginning on the first or second day after the initiation of the experiment, three females from each group were sacrificed, fixed in Heidenhain's susa, and dissected to determine reproductive tract weight. Under either photoperiod regime females fed on live aphids underwent rapid ovarian development which peaked at day 6. Females fed on frozen aphids had reproductive tracts which weighed significantly less than females fed on live prey (Figs. 6A and B).

Effect of Starvation on Flight

Two groups of males and females (n = 30 each) were removed from the 7° C incubator and placed at 24° C (16L:8D photoperiod). One group was fed for 7 days on live aphids and then starved, the other was fed for 3 days on live aphids before starvation. Flight tests were administered daily for 10–12 days. When

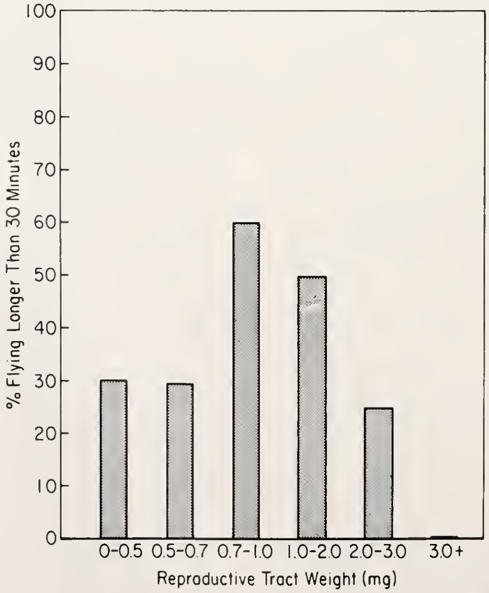

FIGURE 5. Flight activity of female *H. convergens* collected in March (short-day photoperiod) around Austin, Texas with respect to weight of the fixed reproductive tract (n = 38).

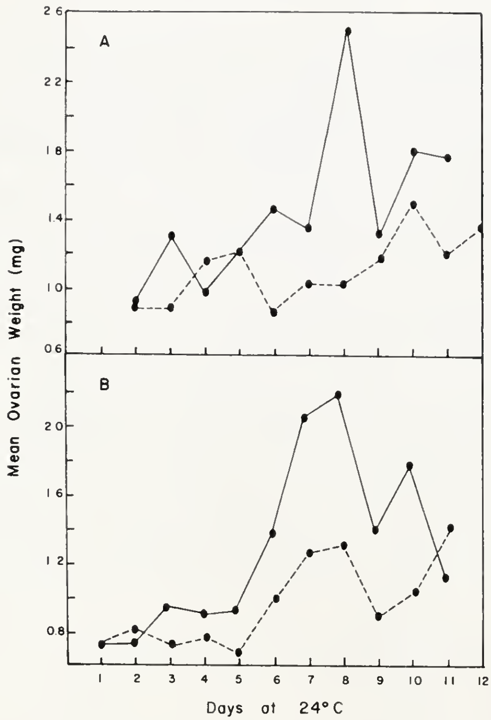

FIGURE 6. Effect of photoperiod and food quality on ovarian weight of *H. convergens* under either a 12L:12D (A) or 16L:8D (B) environmental regime. Each day three females were sacrificed and the weight of the fixed reproductive tract was determined. Solid circles, broken lines indicate females fed frozen aphids while solid circles, solid lines indicate females fed live aphids ($P = 0.025$ for A, $P < 0.001$ for B; Mann Whitney U test).

animals were fed for only 3 days prior to starvation, flight activity was significantly enhanced over that of the fed control group (Fig. 7A). When beetles of either sex were fed for 7 days and then starved, flight activity was not significantly different from that of fed controls at the $P = .05$ level, although it fell near the borderline of significance (Fig. 7B).

Mean ovarian weight of fed controls after 11–12 days was 2.14 mg. Starvation for 5 days after 7 days of food decreased this to a mean of 1.34 mg while females fed for 3 days and starved for 6 days had a mean ovarian weight of 0.69 mg. 33% of the females that were fed for 7 days and then starved oviposited at least once, while none of the females fed for only 3 days prior to starvation laid eggs.

DISCUSSION

In order to investigate the environmental and physiological factors which affect migratory behavior, one must study an insect in which migratory flight can be conveniently assayed in the laboratory and distinguished from "trivial" or appetitive flights. In a group such as the Diptera, in which the animals move about by flying, some ambiguity might exist in interpreting the significance of a long tethered flight. However, since Coleoptera usually move about by walking and fly only occasionally, the tendency to make long continuous flights is likely to indicate the tendency to migrate in this group (Southwood and Johnson, 1957; Southwood, 1962; Dingle, 1965).

Our initial tethered-flight experiments indicated that in *H. convergens* the duration of such flight is a reasonable measurement of willingness to migrate. A beetle that flies 30 min is very likely to fly much longer, often for several hours. Only a portion of the population will make long flights under any circumstances but the ratio of fliers to non-fliers changes throughout the year and the life cycle. The times of greatest percentage of long fliers in laboratory flight tests correspond to the times of population movements to and from the aggregation sites in the field. As is true for many insect migrants, long tethered flights tend to occur most frequently among pre-reproductive *H. convergens* females. For all of these reasons we feel justified in assuming that the tendency to make long tethered flights is an indication of willingness to migrate in this species.

The relationship between age and flight activity is affected by both photoperiod and food quality. Long flights are not made at all for the first 24–48 hr after emergence. After 48 hr post-emergence the tendency to make long flights increases rapidly and peaks from 3–5 days after adult ecdysis. Under short photoperiod and/or poor food quality (frozen aphids) long flights continue for some time in the population. Long day length and high quality food result in a sharp decline in

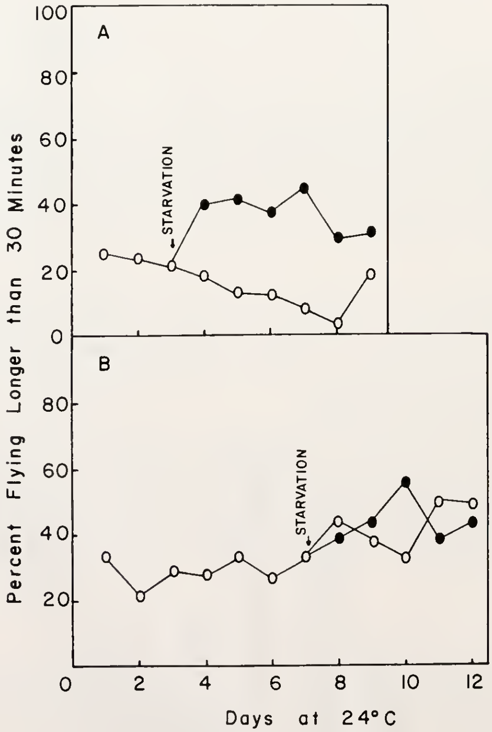

FIGURE 7. Effect of starvation after (A) 3 days of feeding on live aphids or (B) 7 days of feeding on live aphids on flight activity of *H. convergens*. Open circles indicate fed animals, closed circles, starved animals. The difference between the flight performances of starved and control animals is significant at the 0.001 level for A but not significantly different ($P = 0.5$) for B (Mann Whitney U test).

flight after 1 week, by which time oviposition has begun. Photoperiod seems only to influence the duration of the long-flight phase when food quality is optimal. When this is the case, short photoperiod greatly lengthens the period of time in which beetles will make long flights, although the percentage of long fliers in the population is lower when food is optimal than when it is not. When food quality is poor, flight activity is enhanced under either photoperiod regime to quite high levels. Thus the beetles do respond to photoperiod under some conditions, but food quality seems to be the primary cue which triggers migratory behavior. Similarly, ovarian development is dependent upon food quality, photoperiod having little, if any, effect; *H. convergens* can be reproductive under both long- and short-day conditions. This is in agreement with an earlier report by Hagen (1962) and is to be expected from field observations as well: Beetles arrive in the Austin area and begin reproducing in March (short day length) while during the long days of late May, June, and July, beetles may reproduce or migrate depending on prey abundance (Rankin & Rankin, unpublished observations).

Under long days there seems to be a clear oogenesis-flight syndrome (Johnson, 1969) in this species, *i.e.*, ovarian development is inversely correlated with flight activity (Figs. 4A, 5B). While photoperiod does not affect reproduction, it does influence flight activity under some circumstances, as noted earlier. Under short photoperiods, peak flight activity occurs in females with partially developed, rather than completely undeveloped, ovaries. It is interesting to speculate that rising JH titers at the end of the diapause period may be stimulating both flight and ovarian development at this time (Rankin and Rankin, 1980). Furthermore, some flight activity continues in the short-day population until ovarian development is complete (3.0 mg), while it virtually ceases in the long-day animals by the time the reproductive tract has attained 1.5–2.0 mg fixed wet weight (Fig. 5). Thus the "oogenesis-flight syndrome" is less pronounced and flight continues longer in the population under short photoperiods.

Periods of food shortage will cause a cessation of ovarian development in young females and elicit long-flight activity in the population. However, once a female has oviposited, starvation probably does not result in an increase in flight behavior (Fig. 7B). A reproductive female, in response to starvation, would apparently lay what eggs she could and then die. Some physiological commitment to reproduction appears to be made by the time of first oviposition. Our observations in this respect are similar to those of Solbreck and Pehrson (1979) who found that in the Lygaeid, *Neacoryphus bicrusis*, pre-reproductive starvation enhances flight activity as it does in *H. convergens*. Cessation of flight in *N. bicrusis* in the presence of ample food is associated with flight muscle histolysis such that post-reproductive starvation does not elicit further flight. Furthermore, as in our short-day animals, an oogenesis-flight syndrome in *N. bicrucis* is observed only during high reproductive activity.

Beetles that begin reproduction soon after emergence are much shorter-lived than those that delay. For example, by 60 days after emergence, all of the short-day females fed on live aphids were dead while most of the short-day females fed on frozen aphids were still alive. One of the latter group had oviposited one egg, but most had not reproduced. It would seem that once the reproductive machinery is fully stimulated, beetles produce eggs rapidly and then die. If this is true, the large population movements to and from aggregation sites are made largely by pre-reproductive animals, and beetles would typically undergo only one period of aestivo-hibernation. The tendency not to migrate after first oviposition may be less pronounced under short-day conditions or the decision may be made somewhat later. These possibilities are being investigated.

Although several questions concerning the life history of this species remain unanswered, we can surmise a probable sequence of events from our data. Beetles which were newly emerged in June would be exposed to long photoperiods and high temperatures. Summer rainfall conditions, which greatly influence the aphid prey population, are quite variable in Texas. In a wet year, when prey is abundant, these newly emerged beetles would experience a brief period of long flight (2–3 days according to Fig. 3) and then begin reproduction, producing a second generation as they did in 1978 (Rankin and Rankin, unpublished observations). In a dry year, however, such as 1977 (Fig. 2), the aphid prey are very scarce by late June. As Figure 3 indicates, when food quality is poor, the period in which a large percentage of the population makes a long flight is greatly prolonged and the number making long flights is enhanced regardless of photoperiod. Thus shortage of prey would very likely result in the movement of a large portion of the population. Results of flight testing field-collected animals periodically throughout the year support these suggestions. Prevailing winds at this time are such that they would be likely to carry fliers to the west Texas mountains where they subsequently diapause in large aggregations (Crutcher and Halligan, 1967). Initially there may be movement of the beetles around the aggregation area. But as temperatures drop in the fall, activity of any sort diminishes.

In some parts of their range, such as New Mexico and Colorado, beetles have been found under bushes and rocks on mountain tops buried under ice and snow before the spring thaw (M. Rankin, unpublished observations; Douglass, 1930). When such animals are warmed, they will immediately begin to copulate. Similarly, refrigerated animals will quickly begin to mate when they are warmed, even though they will not feed and ovaries are as yet undeveloped. Thus it is likely that female *H. convergens* leave the aggregation sites mated but still reproductively immature. Rising hormone titers may stimulate flight and the onset of reproductive development (Rankin and Riddiford, 1978; Rankin and Rankin, 1980) but the exodus flight is probably made by females unencumbered by large batches of eggs. Feeding on live prey seems to be important in eliciting full ovarian development and according to Figures 3 and 5, long-flight activity would probably continue under the short spring photoperiods, alternating with periods of feeding, until ovarian development was complete. Thus the migrant female would terminate her return flight from the aggregation sites ready to begin immediate reproduction, when her reproductive value is greatest.

Slobodkin (1962) defines reproductive value as "the diminution of future population increase produced by removing a single animal of a given age from a population." It is a measure of sensitivity to natural selection. The time of peak reproductive value, since it represents maximum contribution to future population increase, also indicates that period of the life history at which natural selection exerts its greatest influence (Fisher, 1958). Long flights at this time are likely to have been subjected to strong favorable selection. This may be one reason why there is a high incidence of long-flight behavior in a short-day population even when food is available. Later in the season, when photoperiods are long, the primary cue to migrate is probably food shortage experienced by pre-reproductive adults. Migration at this time precedes a long and somewhat hazardous diapause period. (Our unpublished data indicate that large numbers of beetle carcasses are often found at the aggregation sites in late spring; heavy losses due to nematode infestations have been observed in both New Mexico and Texas aggregations). This migratory behavior might be expected to have been the result of less strong favorable selection than the post-diapause flight. In addition such hazards during the diapause period may strongly favor immediate reproduction when possible. Indeed, migration prior to diapause is much more facultative. During exceptionally wet and thus aphid-rich years, massive aggregations do not occur (Neuenschwander *et al.,* 1975; Rankin and Rankin, unpublished observations) or aggregations do occur but an actively reproducing population is also maintained in aphid-infested areas.

A similar life history strategy is characteristic of the milkweed bug, *Oncopeltus fasciatus.* Dingle (1965) has shown that migratory behavior in this species is post-teneral and pre-reproductive, long flights being terminated as ovarian development is completed. The propagules arrive at their post-migration habitat at the time of peak reproductive value (Dingle, 1965). Rankin (1974) and Rankin and Riddiford (1977, 1978) have shown that the highly adaptive coordination of flight and reproduction in this species is accomplished via the corpus allatum control of both events. Both reproduction and flight are stimulated by juvenile hormone; lower titers of the hormone or shorter exposure time stimulate flight before reproductive development which ultimately inhibits long flights. We suspect a similar situation exists in *H. convergens.* Juvenile hormone stimulates both long flight and reproduction in this species (Rankin and Rankin, 1980) and the data presented in this paper suggest a similar inhibition of long flight when the ovaries are completely developed.

We have not extensively investigated reproductive development in male *H. convergens* migrants. It is likely, however, that migration in males is not solely pre-reproductive. Reproductive activity was not correlated with the tendency to make a long flight and since males mount females readily during the diapause period if they are warm enough to be mobile, it is likely that males are reproductively mature at least by the time they leave the aggregation sites. Males do seem to share the other aspects of the diapause-migration syndrome, however. They show the same variation in proportion of individuals that will make a long flight in the life cycle, during the year, and in response to poor food quality. Males seem to make a similar commitment not to migrate after about 7 days of optimal food (Fig. 7) and they show the same unwillingness to feed during the diapause period. In short, the only difference between the sexes with respect to these characters seems to be that males are reproductively mature during the migration while females are not. It would, of course, be highly advantageous for mating to occur in the large aggregations before dispersal and this is, indeed, often observed among insect migrants (Johnson, 1969; Caldwell and Rankin, 1974).

In summary we conclude that *Hippodamia convergens* is a fairly typical insect migrant in many ways. Long flights seem to be post-teneral and, in females, primarily pre-reproductive and associated with an adult reproductive diapause. Food quality and photoperiod interact in such a way that a spring migratory flight is very likely under any circumstances while the summer migration is much more facultative. Presumably short fall photoperiods would ultimately induce at least some of the population to migrate at that time if a summer exodus had not occurred

It is interesting to note that Hagen (1962) in an excellent review of coccinellid life histories, supports, on the basis of field observations of California *H. convergens,* many of the conclusions we have drawn from our laboratory studies.

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SUMMARY

Hippodamia convergens migrates long distances to and from mountain-top aggregation sites. It undergoes an imaginal diapause associated with a 6–9 month aestivo-hibernation at the aggregation sites. Laboratory tethered-flight tests can be used as an assay for migratory behavior. Beetles that fly 30 min on a tether will nearly always fly much longer.

Long tethered flight is pre-reproductive in females, though apparently not in males. Starvation or poor quality food will greatly enhance migratory behavior. However, the tendency to display migratory behavior is greatly reduced in both sexes after about 7 days of optimum food. Even starvation at that time will not stimulate an increase in long-flight behavior. It would appear that some type of physiological committment to reproduction which excludes or greatly reduces the tendency to migrate is made at that time. Photoperiod has little effect on migratory behavior when food is poor. But under optimal feeding conditions, short photoperiod enhances long flight behavior even among reproductive females.

It would appear that migration to the aestivo-hibernation sites is accomplished primarily by newly emerged (pre-reproductive female) beetles. Strong selection pressure has apparently favored migration away from the aggregation sites in the early spring (short photoperiods) whether or not aphid prey are available, while migration during the summer (long photoperiod) is much more facultative, depending primarily on food abundance.

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THE REGULATION OF ASEXUAL REPRODUCTION AND INDETERMINATE BODY SIZE IN THE SEA ANEMONE *ANTHOPLEURA ELEGANTISSIMA* (BRANDT)

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Certain sea anemones and related anthozoans reproduce asexually to form aggregated or dispersed clones. Such proliferation takes a variety of paths including binary longitudinal fission, transverse fission, pedal laceration, and regeneration from tentacles (reviewed by Sephenson, 1929; Chia, 1976). Little is known about the control of division rates or regulation of the size at which division occurs. The anemone *Haliplanella luciae* divides primarily by longitudinal fission, increasing the rate of fission as feeding rate increases (Minasian, 1976) and dividing at a larger size at higher temperatures and under conditions simulating periodic intertidal exposure (Johnson and Shick, 1977). Smith and Lenhoff (1976) have shown that a small acontiarian anemone will increase its rate of pedal laceration when starved. There appears to be no one common regulator of division across species and no information on the control of asexual proliferation in the field.

The sea anemone *Anthopleura elegantissima* (Brandt), common in the Pacific intertidal zone of North America, divides by binary longitudinal fission to form clonal aggregations (Hand, 1955; Francis, 1973a; Sebens, 1977), which recognize their own members and defend their space against non-clonemates during agonistic encounters (Francis, 1973b, 1976). Division can occur simultaneously among many individuals in a clone (Hand, 1955; Sebens, 1977). Pearse (1974), during a study of photosensitive behavior, noted that individuals divided in both dark and light treatments and that, while a few more divided in the light, the numbers were not significantly different.

Clonal aggregations can be composed of individuals of very different sizes, and mean size of clonal individuals can vary widely across habitats (*e.g.,* 0.8–4.2 cm, Sebens, 1977). Mean individual size increases with decreasing intertidal height, indicating that individuals are probably dividing at a smaller size in the high intertidal. Observation of mapped clones and examination of recent division scars have shown that most division takes place from August to March and that it is primarily the larger individuals in a particular clone that undergo fission (Sebens, 1977). Individuals do not appear to divide until they are at least 1.0–1.2 cm basal diameter, approximately 2 years or more after setttlement. Anemones that have divided are shrinking or plateauing in size during August to March, with the greatest growth occurring from April to July. A theoretical model for optimal size at division and optimal size of colony units in anthozoans has been presented and discussed as it applies to *A. elegantissima* (Sebens, 1979).

Sexual reproduction occurs annually in *A. elegantissima* (Ford, 1964; Sebens, 1977; Jennison, 1977, 1979) and most individuals above 1.2-cm basal diameter are fertile at the peak of the season (July, August; Sebens, 1977). Clone members are all of one sex (Ford, 1964; Jennison, 1977; Sebens, 1977) although a few hermaphrodites have been found at one site (Jennison, 1977). Gonad production begins around January and spawning occurs in August to September (Ford, 1964; Sebens, 1977; Jennison, 1977). Given the high percent fertility (94–100%, Sebens, 1977) of individuals greater than 1.2 cm basal diameter, it is almost certain that some of the same individuals that reproduce sexually undergo fission the following autumn or winter. Very few individuals (< 1%) undergo more than a single fission per season (Sebens, 1977).

The present study was designed to investigate the control of body size at division and the timing of fission in *A. elegantissima*. Specifically, the effects of light, temperature, and feeding regime on weight change and division rate were examined in laboratory aquaria using an experimental group of 1000 anemones. Light regime (irradiance) affects the energetics of *A. elegantissima* through the zooxanthellae which supply part of the anemone's nutrition (Muscatine, 1961). Temperature varies as a factor of season, intertidal height, length of exposure and depth of the tide pools (Dayton, 1971) and feeding rate changes with season, as reflected in the growth and shrinkage rate of anemones in the field (Sebens, 1977).

Materials and Methods

In March, 1976, 1500 specimens of *A. elegantissima* of a full size range were collected at Cattle Point, San Juan Island, Washington (48°27′N, 22°57′W). The anemones were transported to sea-water aquaria at the University of Washington, Seattle, where they were examined for division scars, measured, and kept under observation for 2 weeks. Twenty plastic basins (10 l each) were distributed among constant temperature rooms or provided with aquarium heaters to adjust temperature. All were aerated and half were covered with opaque black plastic sheeting on the top and sides. The other half were covered by clear plastic sheeting, placed under two fluorescent bulbs (40 watt) at 15 cm above the tanks (70–100 microeinsteins per m^2/sec). Five temperatures were chosen (5, 10, 15, 20, 25° C) spanning the range that the anemones normally encounter in intertidal pools (Dayton, 1971). With light and dark treatments at each temperature, and fed and starved groups for each of those 10 treatments, 20 separate treatments (aquaria) resulted.

Fifty anemones (undamaged and covering the size range) were placed in each basin filled with filtered (particles $\geq 20\ \mu m$ removed) sea water. Feeding was every other day for half of the treatments (500 anemones). Pieces of mussel (*Mytilus californianus*, tissue fragments 0.2–0.5 g wet weight) from frozen stocks were fed with forceps to each anemone. The following day all pieces of egested or rejected food were removed by suction with a large plastic syringe. Water was changed every fourth day (after every second feeding) and most of the developing algal layer on the basin surfaces was removed at that time with a paper towel.

Anemones were observed daily, noting any that began a division or appeared to do so. Early potential dividers showed elongation of the pedal disc (most did not go on to divide). Intermediate potential dividers elongated the oral aperture as well (most divided). Actual dividers had begun separation of the halves. Post dividers had completed the division, showing new division scars. Sizes of anemones at any stage of potential division were measured (length and width of pedal disc) and their positions were drawn on data sheets with oriented maps of each tank. All anemone sizes were measured at 0, 34, and 50 days of the experiment.

A plastic-screened enclosure holding ten anemones (approx. 1.5-cm diameter each) was placed in the center of each basin. This constituted the weight monitored group. Anemones were identified with small neutral red dye spots (Sebens,

1976) and were easily removed from the smooth plastic surface. They were weighed in sea water (on a torsion balance) by the reduced weight method (Zeuthen, 1948; Muscatine, 1961) followed by a rapid weighing in distilled water to calculate any change in anemone tissue density during the course of the experiment. Anemones were hung on fine stainless steel wire suspended from a torsion balance in a beaker of seawater (from a filtered stock kept in the dark for the duration of the experiment). Treatments ran for a total of 60 days (April–May, 1976).

RESULTS

Weight change

Weight changes of specimens of *Anthopleura elegantissima* over a 30-day period, expressed as percent change, were submitted to an analysis of variance (single classification, Model I) to ascertain the effect of all treatment variables. Means, 95% confidence limits for the mean, and the analyses of variance were run using both the original data and arcsine transformations of the data suggested for percentages and ratios (Sokal and Rohlf, 1969). The transformation used was as follows: $\theta = \text{arcsine}\ ((P + 100)/200)^{0.5}$, where p is the original percent weight change.

TABLE I

Descriptive statistics for 30-day percent weight change of Anthopleura elegantissima. *$X\%$, s, L_1, L_2 are mean, standard deviation, and upper and lower 95% confidence limits of the mean for percent weight change data. The equivalent statistics were calculated for arcsine transformed data, where $0° \leqslant \theta \leqslant 90°$. X_{arc}, L_1, L_2 are mean, and upper and lower 95% confidence limits for percent weight change, calculated by back-transforming the mean and limits determined for θ. Treatments are noted by temperature (5°, 10°, 15°, 20°, and 25° C), feeding regime (fed (F), starved (S)), and illumination (light (L), dark (D)).*

Treatment	Percent change			Percent change (arcsine)	
	$\bar{X}\%$	s %	L_1 L_2	$\bar{X}_{arc}$	L_1 L_2
5° SD	−23.4	6.2	(−29.1, −17.7)	−23.5	(−29.1, −17.8)
5° FD	− 1.9	16.9	(−17.5, +13.7)	− 1.9	(−17.5, +13.7)
5° SL	−11.4	10.0	(−20.6, − 2.2)	−11.4	(−20.6, − 2.2)
5° FL	+24.5	34.0	(− 6.9, +58.5)	+32.1	(−18.2, +74.3)
10° SD	−31.4	6.0	(−37.0, −25.8)	−31.5	(−37.0, −25.8)
10° FD	+15.8	21.0	(− 3.5, +35.2)	+16.3	(− 4.0, +35.8)
10° SL	−12.9	9.4	(−21.5, − 4.2)	−12.9	(−21.6, − 4.2)
10° FL	+48.8	37.3	(+14.4, +83.2)	+51.5	(+13.0, +81.5)
15° SD	−28.5	15.9	(−43.1, −13.9)	−28.9	(−43.6, −13.5)
15° FD	+ 0.9	21.4	(−18.9, +20.7)	+ 0.9	(−19.0, +20.9)
15° SL	−23.9	8.2	(−31.5, −16.4)	−24.0	(−31.5, −16.4)
15° FL	+15.7	8.1	(+ 8.3, +23.2)	+15.8	(+ 8.3, +23.2)
20° SD	−39.7	8.9	(−47.9, −31.5)	−39.9	(−47.9, −31.5)
20° FD	−27.5	12.1	(−38.6, −16.3)	−27.7	(−38.8, −16.2)
20° SL	−42.5	10.0	(−51.7, −33.2)	−42.7	(−51.7, −33.2)
20° FL	−37.2	13.0	(−49.2, −25.2)	−37.6	(−49.5, −25.0)
25° SD	−44.0	22.3	(−64.6, −23.4)	−45.0	(−64.0, −23.7)
25° FD	−41.6	10.2	(−51.1, −32.2)	−41.9	(−51.1, −32.2)
25° SL	−41.9	8.6	(−49.8, −34.0)	−42.1	(−50.0, −33.9)
25° FL	−46.5	5.6	(−51.7, −41.3)	−46.6	(−51.8, −41.3)

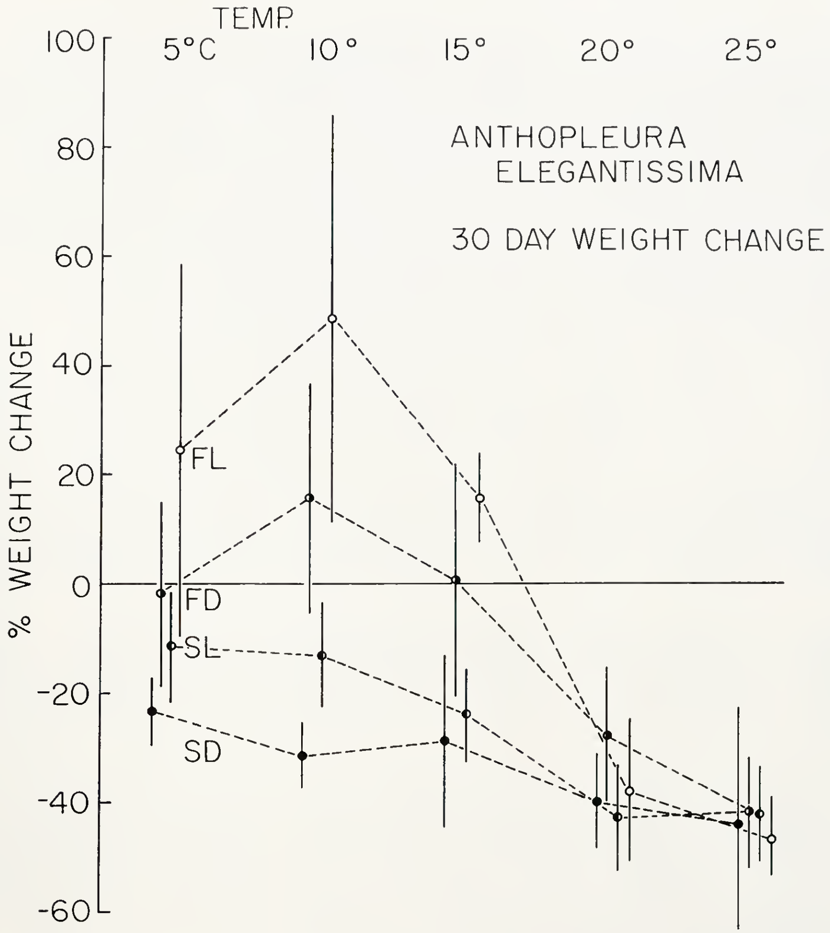

FIGURE 1. Weight change of groups of seven specimens of *A. elegantissima* in each experimental treatment in the laboratory (± standard deviation) expressed as percent original weight.

Table I compares the mean, standard deviation, and 95% confidence limits for the mean as calculated for the original percent data and from the transformation, θ. Only the values for the 5° C group fed in the light and the 10° C fed in the light gave somewhat different estimates by the two methods.

Assumptions made in performing an analysis of variance are met to some extent by the data. Individuals used in the weight change experiment were a blind choice from all available individuals. The experimental design was such that aquaria were identical in construction and their positions were independent with respect to external influences. The assumption of homogeneity of variances (homoscedasticity) (Sokal and Rohlf, 1969) is met in some of the comparisons but not in others (F-max test). Analysis of variance was carried out on all the data, and on subgroups defined by feeding regime, illumination, temperature and finally as 1 to 1 comparisons of groups within each temperature. Calculations were done on an IBM 360 computer using the SPSS subprogram ANOVA (Nie, Hull, Jenkins, Steinbrenner and Bent, 1975). Two-way analysis of variance (Model I)

TABLE II

Two-way analysis of variance on 30-day percent weight change of Anthopleura elegantissima. *"Data group" denotes the portion of the total treatments used in the analysis. "Factors" are the subgroups to be compared in Model I ANOVA. N is the number of data points per subgroup and d.f. are degrees of freedom. Sums of squares are divided into the interaction sum of squares (SS_2) and total (SS_t). Mean squares are given as interaction mean square (MS_2) and total (MS_t). F_s is given for percent change data, with its significance level. F_s, determined by carrying out the same ANOVA on arcsine transformed percent change data, is also given with its significance level. * denotes significance at the $P < 0.05$ level, ** at the $P < 0.01$ level, *** at the $P < 0.001$ level. Subgroups are denoted by the following: feeding regime (Fd) including fed and starved groups, illumination (I) including light and dark groups, and temperature (T) including treatments at 5°, 10°, 15°, 20°, and 25° C.*

Data group	Factors	N	d.f.	SS_2	SS_t	MS_2	MS_t	F_s	$F_{s\theta}$	sig. F_s	sig. $F_{s\theta}$
All	I, Fd	140	1	0.2	127.8	0.2	0.9	0.3	0.5	0.604	0.494
All	T, Fd	140	4	13.2	127.8	3.3	0.9	10.3	7.5	0.000***	0.000***
All	T, I	140	4	5.0	127.8	1.2	0.9	2.3	2.2	0.060	0.070
Fed	T, I	70	4	4.8	88.9	1.2	1.3	2.9	2.4	0.029*	0.064
Starved	T, I	70	4	1.0	17.1	0.2	0.2	1.9	1.6	0.129	0.176
Light	T, Fd	70	4	9.9	84.8	2.5	1.2	8.0	5.0	0.000***	0.002***
Dark	T, Fd	70	4	4.1	40.0	1.0	0.6	12.3	12.3	0.000***	0.000***
5°	I, Fd	28	1	0.4	18.2	0.4	0.7	0.9	0.8	0.347	0.394
10°	I, Fd	28	1	0.3	36.0	0.3	1.3	0.7	1.1	0.416	0.298
15°	I, Fd	28	1	0.2	14.3	0.2	0.5	0.9	0.9	0.359	0.396
20°	I, Fd	28	1	0.1	3.9	0.1	0.1	0.7	0.6	0.414	0.429
25°	I, Fd	28	1	0.1	4.4	0.1	0.2	2.8	2.7	0.475	0.497

was also used to examine interaction effects between the three treatment variables. All analyses were carried out on the original percent change data (made positive by addition of a scalar, 100), and on the arcsine transformed data. Results of the two-way analysis are given in Table II with the F values and their significance levels supplied by the SPSS program. Complete results of the one-way analysis are available from the author upon request.

The results of the analyses of variance will be discussed as they relate to each treatment variable: Temperature significantly affected weight change when all data were subjected to the analysis ($P < 0.001$), within each of the feeding regimes ($P < 0.001$) and within each of the illumination regimes ($P < 0.001$). Illumination did not significantly affect weight change when all data were subjected to the analysis, or when only the fed or starved groups were used. It did affect weight change significantly only in the 5° C temperature group and in the 5° and 10° C starved groups when temperature groups were broken down for one-to-one comparisons. Feeding regime had a significant effect on weight change when all data were used in the analysis ($P < 0.001$), within the light ($P < 0.001$) and dark ($P < 0.001$) groups and in each of the temperature groups except those at 20° and 25° C. When temperature groups were broken down for one-to-one comparisons, feeding regime had a significant effect in all groups except those at 20° and 25° C.

Two-way analysis of variance indicated a significant interaction effect ($P < 0.001$) between temperature and feeding regime within all groups tested. A significant interaction also occurred between temperature and illumination in the fed group ($P < 0.05$). No significant interaction was indicated between feeding regime and illumination when all data were used, or in any of the temperature

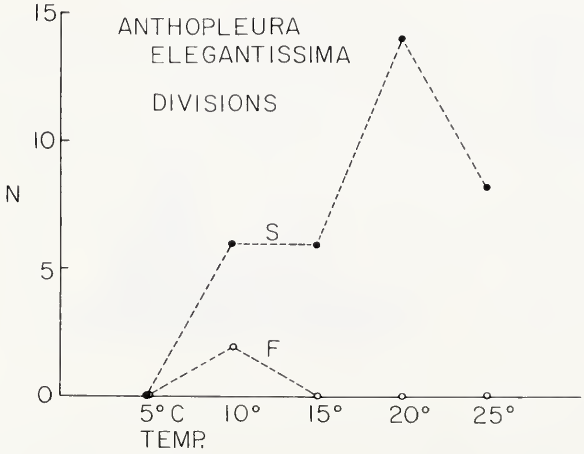

FIGURE 2. Numbers of divisions in each treatment during laboratory experiments with *A. elegantissima.* Starved (S) and fed (F) groups are compared at each experimental temperature. Number of divisions are those that occurred over the total 60-day period. Maximum number of divisions was 50 per treatment.

treatments. The last result is interesting since feeding and illumination both affect energy intake, and can be presumed additive, while temperature affects food assimilation, feeding behavior, and metabolic cost and would not be presumed to have an additive effect with food or illumination.

Differences between light and dark groups agree well with Muscatine's (1961) measurements of weight loss in starved specimens of *A. elegantissima* with and without zooxanthellae. At 20° and 25° C all groups lost weight at a comparable rate, independent of treatment. At the lowest temperature, 5° C, anemones grew or shrank less than at 10° and 15° C. At that low temperature, activity was reduced and food was often rejected or egested with little apparent change. If symbiotic algae (zooxanthellae) produce oxygen more rapidly at higher temperatures, but also metabolize more of their photosynthetic products, zooxanthellae would be a liability and anemones would lose weight more rapidly because of increased metabolic rate. Metabolic rate increases with oxygen tension and temperature for anemones (Sassaman and Mangum, 1972; Sassaman 1973, 1974; Brafield and Chapman, 1967; Beatties, 1971). At 25° C most of the anemones ejected their algal symbionts after two weeks. Pearse (1974) was able to produce algae-free specimens for experimental use by keeping them at similar raised temperatures. Goreau (1964) and Reimer (1971) have noted that various anthozoans expel zooxanthellae during extreme stress. This result suggests that the intracellular symbionts are rejected when their presence begins to subtract from, rather than add to, the host's energetic budget.

Density change of anemone tissue was monitored by weight difference in two fluids (sea water and distilled water). The reduced weight technique is accurate for measuring weight change only if tissue density does not change substantially during the experiment (*e.g.,* if only lipid were lost, it would make tissue more dense). Weight (g) in sea water (W_s) is given by: $W_s = (D_t V_a) - (D_s V_a)$ where D_t is density (g/cm^3) of tissue, D_s is the density of distilled water and V_a (cm^3) is volume of anemone tissue, equal to water displaced when the anemone is immersed for weighing. Weight in distilled water (W_f) is: $W_f = (D_t V_a) -$

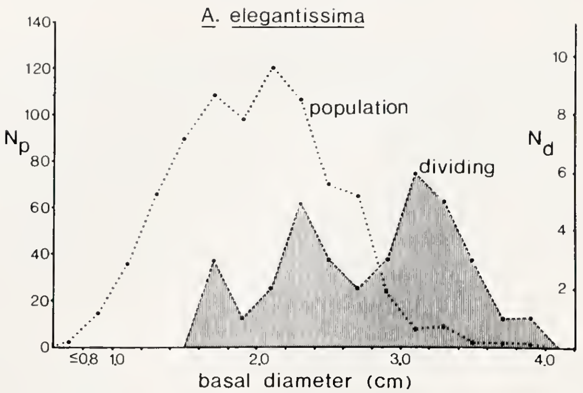

FIGURE 3. Sizes of specimens of *A. elegantissima* dividing (N_d scale) during laboratory experiments. Size-frequency distribution of the entire experimental population (N_p scale). Mean diameter of the experimental population was 2.0 ± 0.5 cm s.d. ($N = 730$). Individuals that divided had a mean diameter of 2.9 ± 0.5 cm s.d. ($N = 34$).

($D_f V_a$) where D_f is the density of distilled water. The ratio of W_s to W_f is: $W_s/W_f = (D_t - D_s)/(D_t - D_f)$ where the V_a term is removed. The ratio will obviously change if D_t changes during weight change of the anemone. The value of W_s/W_f at the beginning of the experiment was 0.54 ± 0.11 s.d. for the groups at 5° C fed and starved in the dark (N = 18) and 0.47 ± 0.07 s.d. for the same groups after 34 days. No significant change had occurred in the ratio (Student's t-test, t = 2.0, not significant). W_s/W_f does not differ from a normal distribution in the groups before or after weight loss (Chi-square test, d.f. = 19).

Frequency of asexual division

Division frequencies were not significantly different in light and dark treatments (19 light, 17 dark) but were very different in the fed and starved groups (34 starved, 2 fed) (Fig. 2). A Chi-square test of number of individuals dividing per group of 50 showed significance for all fed vs. all starved groups ($\chi^2 = 28.44$, d.f. = 1, $P < 0.0001$), for different temperature treatments within the starved group ($\chi^2 = 14.82$, d.f. = 4, $P < 0.01$) but not for different temperatures within the fed group. Within the starved groups, division frequency increased with temperature to 25° C, where divisions were fewer. At 25° C many of the anemones died and others showed signs of physiological stress (everted actinopharynx, reduced sensitivity to prodding) and the entire division process may have been impaired. At 20° and 25° C, even though fed groups were actually losing weight, they were not dividing. Possibly the presence of food inhibited division while increased metabolic rate stimulated division only when individuals were not feeding.

Only the larger anemones divided during the experiment (Fig. 3). The experimental population had a mean initial individual diameter of 2.1 ± 0.6 cm s.d. while the anemones that divided had a predivision diameter of 2.9 ± 0.5 cm s.d. Anemones that did not divide during the course of the experiment began at a mean size of 2.0 ± 0.5 cm s.d. Dividing anemones were significantly larger than those that did not divide (Student's t-test, $P < 0.001$). This agrees well with the sizes of anemones that divided (1.9 ± 0.4 cm s.d.) and did not divide (1.5 ± 0.4 cm s.d.) in the field (Sebens, 1977), determined by comparing those with and without division scars (signficantly different by Student's t-test, $P < 0.001$). The sizes of anemones that divided during the experiment were significantly smaller at 20° C (2.0 ± 0.5 cm s.d.) and 25° C (2.0 ± 0.6 cm s.d.) than at 10° C (2.5 ± 0.6 cm s.d.) and 15° C (2.6 ± 0.5 cm s.d.). (Student's t-test, $P < 0.05$). Sizes of anemones dividing at 20° and 25° C were not significantly different from each other nor were those at 10° and 15° C.

Increased metabolic cost due to raised temperature may have stimulated division rate in the experimental aquaria. In the field, largest individual size within a clone correlates with observed temperature. Figure 4 compares maximum temperatures reached by anemones in 15 tide pools exposed at low tide on a single day with the mean size of the ten largest individuals in each pool. All pools (Iceberg Point, Lopez Island) were on a flat bench within (0.09 m) intertidal height, and so experienced the same immersion time for feeding. The pools thus differ primarily in temperature while exposed, influencing metabolic cost, which increases with temperature. In fact, pools which reach lower temperatures due to shading or depth have the larger anemones. That is, division probably occurred at a smaller size in pools that reach higher temperatures. High-intertidal anemones experience generally greater temperatures when exposed than do those in the low intertidal. Consequently, the former are smaller at both sites in the San Juan Islands (Sebens, 1977).

DISCUSSION

Asexual division is inhibited when *A. elegantissima* is fed continuously. Such proliferation occurs more rapidly and in a larger fraction of the population as ambient water temperature increases. Starvation, defined as weight loss, is not in itself sufficient stimulus to initiate the division process. If shrinking individuals are given food, even though it may not be efficiently assimilated (as at 20° and 25° C) (Fig. 1), division still does not occur. Light or darkness has no significant effect on the division process although it has a marked effect on weight

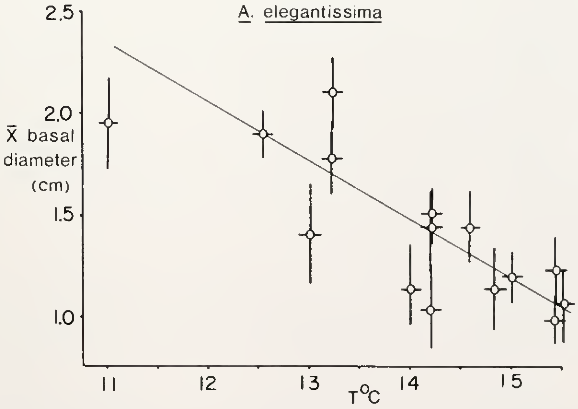

FIGURE 4. Mean basal diameter (± 95% confidence interval) of *A. elegantissima* in high tidepools (+ 1.8 m to + 1.9 m) at Iceberg Point, Lopez Island, plotted against maximum temperature (± range) reached by the pool at a given low tide. Mean basal diameters are the mean of the ten largest anemones in each pool. The regression line is: basal diameter (mm) equals $-2.3T(° C) + 46.4$ with $r = 0.77$.

change. Finally, it appears that only the larger members of the population undergo fission, as in the field (Sebens, 1977).

Weight change during the experiment was significantly affected by temperature, feeding regime, and illumination (Fig. 1). Muscatine (1961) found that *A. elegantissima* lost weight less rapidly in the light when it had zooxanthellae, thus implicating the zooxanthellae as a positive factor in the anemone's energetic regime. This result is supported here in a slightly different manner. All anemones had zooxanthellae at the beginning although many of the anemones at 25° C egested them during the course of the experiment. Weight gain in fed anemones was greater and weight loss during starvation less when given a high level of irradiance, again pointing to a significant nutritional role for the zooxanthellae.

The effect of illumination and feeding regime changed at 20° and 25° C, at which point all treatment groups lost weight rapidly. Zooxanthellae, mostly retained at 20° C, are no longer an advantage and may in fact become an energetic burden. At 5° C, prey digestion or assimilation efficiency must be reduced since weight gain was much less than at 10° C. However, metabolic rate must also be lower and thus weight loss in starved groups was less at 5° C than at any of the other temperatures. Temperatures do get this low during winter exposures in the intertidal (Dayton, 1971) and the anemones may be energetically shut down for the season. Size changes in the field support this contention (Sebens, 1977). Continuous exposure to 5°, 20°, or 25° C is, however, outside of the range experienced by *A. elegantissima*.

The most interesting weight changes occurred at 10° C, a common ambient temperature for the anemones during much of their growth and reproductive season (Dayton, 1971; Sebens, 1977) on the Washington coast. The separation of the treatment effects was the greatest and most significant and the zooxanthellae thus had their greatest effect on anemone energetics in both fed and starved individuals. Feeding produced the greatest weight gain at this temperature even though starvation caused shrinkage at a rate slightly greater than at 5° C. The light level used, well below that which can be encountered in the intertidal (yet constant over 24 hr), produced a weight gain in fed animals greater than twice that in the dark and a weight loss during starvation less than half that in the dark.

The results of the laboratory experiments agree well with the field observations on individual size within clones and timing of division. Division occurs during the time of year when temperatures are relatively low (6°–10° C), prey is least available, and there is time for the necessary internal reorganization that must occur before the next feeding period. During the months of August through December, individuals are generally shrinking, as measured by basal diameter (Sebens, 1977). Both mapped individuals in monitored clones and quadrat samples of the population at large showed this trend (six sites).

Starvation during the autumn and winter months could thus be a cue to initiate the division process, although prolonged lack of prey during any time of the year may do so in some members of the population which have wandered into particularly poor locations. The field studies showed a few dividing individuals even in the summer months. Individuals used for this set of experiments were collected during the winter when much of the population does undergo fission and when the sexual reproduction cycle is just beginning; hence gonads were not yet a confounding factor. It is not known whether fully developed gonads can act as a deterrent to division.

By contrast, other anemone species (*e.g.*, *Haliplanella luciae*) can undergo fission repeatedly and are probably not tied to an annual cycle of the same type as is *A. elegantissima* (Minasian, 1976; Johnson and Shick, 1977). An unidentified acontiarian anemone (Smith and Lenhoff, 1976) and *Metridium senile* (Davis, 1920, and personal observation) form pedal lacerates repeatedly and show no apparent seasonality. *Anthopleura elegantissima* shares a similar morphological form of longitudinal fission with *Haliplanella luciae* yet the two respond very differently to ambient conditions. *H. luciae* divides more frequently when fed (Minasian, 1976) and divides at a larger size at higher temperatures (Johnson and Shick, 1977), exactly the reverse of *A. elegantissima*. The control of division is thus highly species-specific and very likely adapted to the ecological conditions over the range of habitats occupied by a particular species.

The present set of experiments gives some indication as to how control of size may come about. *A. elegantissima* is usually limited to a single division per individual per year. Mean size of individuals within clones thus will change with habitat if some habitat-dependent threshold size must be attained before division. In the laboratory experiments, more individuals per treatment divided as temperature increased from 5°–20° C. Individuals dividing at the lower temperatures were generally larger than those dividing at the higher temperatures. If the decision to divide is based on energetics (*e.g.,* they will divide at a smaller size if losing weight more rapidly), then a single annual division could produce the observed pattern of variable size. This situation has been used as an example of size regulation in a theoretical model of indeterminate growth (Sebens, 1979).

Some closely related anemones never divide asexually (*e.g., Anthopleura xanthogrammica* (Brandt), Sebens, 1977) and a variant of *A. elegantissima* in California which grows to a large size (at least 15-cm basal diameter) appears not to divide (Sebens, 1977; Francis, 1979). According to the results of these experiments, the large variant, if indeed the same species, could be produced by continuous feeding, removing the starvation cue for division. Whether or not this is actually the case, it is clear that there are situations where division is either impossible or somehow maladaptive. Elsewhere Sebens (1979) has shown that a large solitary individual can be energetically favored when the prey resource consists of large, relatively rare, prey items. Division would produce smaller individuals with greater total feeding surface but unable to use the large prey items. Both *A. xanthogrammica* and the large variant of *A. elegantissima* occur lower in the intertidal than the clonal *A. elegantissima* and both feed on large mussels (*Mytilus californianus*) while smaller *A. elegantissima* feed on a variety of intertidal invertebrates and zooplankton of relatively small size (Sebens, 1977). Inhibition of division in the case of large prey specialists could be facultative (a single species could be clonal or solitary depending on prey and habitat) or obligate, losing the ability to reproduce asexually over evolutionary time (*e.g., A. xanthogrammica*). A facultative case may occur in *A. elegantissima* and in a co-familial anemone in the Chilean intertidal, *Phymactis clematis* (Drayton) which produces both small clonal and large solitary individuals (Stotz, 1977; Sebens and Paine 1978). The ability to form clonal aggregations or dispersed clones may be advantageous in space occupation and defense (Francis, 1973a).

Asexual reproduction by fission shares more aspects with colony formation than with reproduction by small propagules. Williams (1975) describes this type of reproduction in his "strawberry-coral model" as a means of occupying nearby habitat space in an area already proven suitable for the "parental" genotype. This appears to be a reasonable model for the clonal aggregations of sea anemones which colonize an area as a sexual propagule (planula larva) and then fill available space through asexual means. Such anemones usually produce gonads and sexual products annually, devoting as much of their relative biomass to this activity as do species which do not divide (Sebens, 1977). With external fertilization and planktonic larvae (Siebert, 1974), *A. elegantissima* is essentially unable to colonize even nearby areas with sexual offspring. All sexual propagules are used in long-range dispersal and all asexually produced individuals are retained in the same habitat if not always in the same aggregation.

Binary fission producing roughly equal daughter individuals is not the only option taken by anemones. Pedal laceration produces multitudes of small tissue fragments, each of which becomes a new anemone much smaller than the parent. Calow *et al.* (1979) have discussed the advantages of equal and unequal fission in freshwater flatworms where one part receives the pharynx and the other must regenerate everything anew, much the same as in pedal laceration where a lacerate must regenerate all internal structures and tentacle crown before it can begin feeding. This results in one relatively low risk and one or more high risk offspring, which may be better at times than two resulting units neither of which can feed for awhile. If vertical size were an advantage in either intraspecific or interspecific competition, this arrangement could be very important.

As is true for morphological attributes, the selective pressures on asexual proliferation can be numerous, even in a relatively simple process such as binary fission. Factors affecting timing and size at division have been considered but these are only a few of the potential variables involved. This form of asexual multiplication has not been represented as an alternative to sexual reproduction. On the contrary, it appears that the proper timing of division and regulation of indeterminate body size acts to maximize the energetic budget of *A. elegantissima* such that the output of gametes per clone at the end of the growing season is as great as possible.

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SUMMARY

The sea anemone, *Anthopleura elegantissima,* forms distinct clonal aggregations in the field by longitudinal fission (Hand, 1955, Ford, 1964, Francis, 1973a, b, 1976, Sebens, 1977). Field observations indicate that division takes place most

often in the fall and winter when individual anemones are decreasing in size (Sebens, 1977). Mean size of clonal individuals decreases with increasing intertidal height (Sebens, 1977) and with increasing tidepool temperature at a given height.

In the laboratory, asexual division is inhibited when *A. elegantissima* is fed continuously. Such division occurs more rapidly and in a larger fraction of the population as ambient water temperature increases. Starvation, defined as weight loss, is not in itself sufficient stimulus to initiate the division process. Shrinking individuals, given food, do not divide even though that food may not be efficiently assimilated (as at 20° and 25° C). Strong light or darkness has no significant effect on the division process although it has a marked effect on weight change. Finally, it appears that only the larger members of the population undergo fission, as in the field (Sebens, 1977).

Weight change in experimental groups is significantly affected by temperature, feeding regime, and illumination. Evidence that zooxanthellae are a significant positive factor in the anemone's energetic regime is supported. All anemones had zooxanthellae at the beginning although many anemones at 25° C egested them during the course of the experiment. Weight gain in fed anemones was greater, and weight loss during starvation less, when given illumination than in darkness.

The effect of illumination and feeding regime changed at 20° C and at 25° C, at which point all treatment groups lost weight rapidly. Analysis of variance indicates significant interaction between temperature and feeding regime, and between temperature and light (in the fed group) as factors affecting weight loss or gain. Light and feeding regime do not show significant interaction.

The results of laboratory experiments support the hypothesis that seasonal control of longitudinal fission is affected through the energetic regime, commencing during periods of starvation. More rapid weight loss and lesser feeding time in high intertidal pools may result in division at a smaller size and thus clones where mean individual size is smaller than in the low intertidal.

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GEOGRAPHICAL VARIATION IN THE STRENGTH OF THAIDID SNAIL SHELLS

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One important selective pressure on molluscan shells is imposed by shell-breaking predators. Direct evidence for this biologically caused mortality comes from field studies of predation (Kitching, Muntz and Ebling, 1966; Kitching and Lockwood, 1974). The intensity of shell breaking is known to be greater in the tropics than in the temperate zones (Vermeij, 1979a). Correspondingly, tropical shells show greater development of traits that confer protection against crushing than do species in colder waters; these features include a globular shape (low spire), strong sculpture and a narrow or occluded aperture (Vermeij, 1978; Palmer, 1979).

In this paper we examine the mechanical resistance of shells to forces similar to those imposed by crushing predators such as fishes and crabs. Using the latitudinally widespread family Thaididae we show that tropical shells are considerably more resistant to cracking than temperate ones, and that this difference is to a considerable extent associated with differences in shell build (the size and shape of the shell) and microstructure.

MATERIALS AND METHODS

Specimens of *Thais* (= *Nucella*) *lapillus* were collected by J. D. C. at Scarborough, Yorkshire, England, during 1978. Other species were collected by G. J. V. from various parts of the world from 1969 to 1977. The specimens were first boiled briefly until the soft parts could be removed with the aid of a needle, and were then dried at room temperature. They were thoroughly rewetted before testing. Curry (1979) has shown that such treatment is unlikely to have a serious effect on shell strength. The following shell measurements were made: dry mass M; length L (distance from apex to tip of siphonal canal); maximum breadth B. A measure of volume was estimated as $V = L \times B^2$. The acuteness of the shell was expressed as the ratio L/B. The thickness of the shell wall was considered, for comparative purposes, to be represented by the mass to volume ratio (M/V); for convenience this is expressed as $(g/mm^3) \times 10^5$.

Each shell was placed between the metal platens of a Howden compressive testing machine, the apertural side downwards and the dorsum of the shell touching the upper plate. The platens were brought together at the rate of 1 mm per minute. The shells broke within a few seconds. The maximum load borne by the shell was recorded; we call this value the strength. The method of testing we used corresponds reasonably well to the type of force exerted by rays, puffers, and crabs that crush the shell, but not to the peeling action exerted by many crabs (Shoup, 1968). Although not specifically dealing with the latter mode of attack, the method of testing used gives an idea of the general robustness of the shells.

The fracture surface of at least one specimen from each species was examined under a scanning electron microscope. All species showed crossed-lamellar struc-

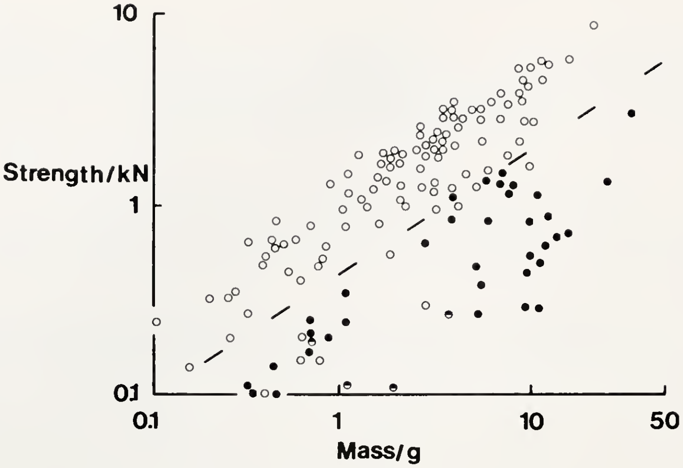

FIGURE 1. Relationship of strength and mass of shells. Open circles—tropical sites; solid circles—temperate sites; half-filled circles—Dakar. The interrupted line is log load (knN) = − 0.3 + 0.67 (log mass (g)).

ture, as would be expected in the Thaididae (Bøggild, 1930). However, in a few specimens it was ill defined, and could be seen only with difficulty. In such cases several specimens of the same species were examined to see whether this was a general phenomenon, or whether it was restricted to a single individual. It seemed always to be characteristic of the species. Some species had a very characteristic layer of calcite on the outer surface of the shell, which looked almost structureless under the scanning electron microscope. Again, according to Bøggild (1930), this is not unexpected. In two species this layer made up a substantial proportion of the whole thickness of the shell: about 30–40%. In the other species the layer was very thin, and has been ignored in the discussion.

The question of whether a shell comes from a tropical or a temperate faunal zone is in most cases easily decided. Only for Dakar (on the bulge of Africa) is there some doubt (Briggs, 1974). Dakar has a minimum sea-surface temperature of about 19° C. This value is intermediate between the minima of other sites that are clearly tropical or temperate.

RESULTS

Figure 1 shows the relationship between strength and shell mass. Note both ordinate and abscissa are on a log scale. Temperate shells are shown by solid circles, tropical shells by open circles. There were 36 shells of *Thais lapillus*. In order that they should not dominate the diagram visually we chose seven randomly. However, *all* shells of this species are included in the calculations and discussion. Expressing shell strength in relation to mass shows, in effect, the amount of protection that can be obtained from a unit mass of shell. In general, if a shell is stronger per unit mass this implies that it is thicker-walled. This is certainly true for *Thais lapillus* studied by Kitching, Muntz and Ebling (1966).

In general, the tropical shells lie above the temperate ones. It is also clear that, although the lower boundary is ill-defined, the upper boundary is quite sharp. This may indicate the maximum strength that can be achieved by a thaidid shell

TABLE I

"Minimum sea temperature"—Data taken from maps in Sverdrup, Johnson and Fleming (1970), except for Eilat (data supplied by Israeli Embassy, London, personal communication) and Panama (data from U.S. National Ocean Survey, 1970).

"Tropical"—Checks indicate certainly tropical, crosses certainly temperate, question marks intermediate. "Above" and "Below"—Number of specimens above and below the interrupted line in Figs. 1 and 2. "Acuteness"—The mean ratio of length to breadth of the specimens. "Thickness"—The mean value of the mass/volume ratio of the specimens.

"Structure"—Checks indicate clear crossed-lamellar structure, crosses indicate indistinct structures; C indicates a thick layer of calcite on the outside of clear crossed-lamellar structures. After the locality name, P indicates Pacific or Indopacific, and A indicates Atlantic.

Species	Locality	Min. Sea Temp.	Tropical	Above	Below	Acuteness	Thickness	Structure
Cymia tectum	Panama P	27	√	2	0	1.20	28	√
Drupa arachnoides	Guam P	27	√	7	0	1.11	38	√
Drupa morum	Guam P	27	√	5	0	1.09	43	√
Drupa ricinus	Guam P	27	√	6	0	1.12	38	√
Drupella alata	Guam P	27	√	2	0	1.85	55	√
Morula fiscella	Guam P	27	√	2	0	1.60	32	√
Morula granulata	Hawaii P	27	√	7	0	1.45	37	√
Morula granulata	Guam P	27	√	9	0	1.48	52	√
Morula granulata	Palau P	27	√	6	0	1.38	55	√
Morula granulata	Eilat P	20	√	5	0	1.51	48	√
Morula uva	Guam P	27	√	2	0	1.41	44	√
Thais deltoidea	Curaçao A	25	√	6	0	1.31	33	√
Thais deltoidea	Panama A	27	√	3	0	1.35	41	√
Thais hippocastanum	Guam P	27	√	2	0	1.23	28	√
Thais kiosquiformis	Panama P	27	√	1	0	1.32	19	√
Thais melones	Panama P	27	√	7	0	1.31	53	√
Thais triangularis	Panama P	27	√	2	0	1.16	37	√
				Mean		1.35	40.1	
Acanthina brevidentata	Panama P	27	√	4	1	1.54	36	√
Thais coronata	Ivory Coast A	24	√	2	2	1.27	37	C
Thais haemastoma	Ghana P	24	√	8	1	1.46	33	√
Thais haemastoma	Costa Rica P	26	√	4	4	1.36	26	√
Thais lapillus	England A	6	x	1	35	1.59	50	x
				Mean		1.44	36.4	
Acanthina calcar	Chile P	14	x	0	4	1.35	41	x
Purpura patula	Curaçoa A	25	√	0	2	1.45	18	√
Thais chocolata	Peru P	17	x	0	2	1.34	28	C
Thais haemastoma	Dakar A	19	?	0	4	1.62	27	√
Thais haemastoma	Peru P	19	x	0	12	1.43	21	√
Thais lamellosa	Washington P	9	x	0	10	1.75	30	x
				Mean		1.49	27.5	

built of crossed-lamellar structure while still allowing some living space inside. The tropical scatter of strengths follows a slope of strength ∝ mass$^{\frac{2}{3}}$ fairly closely, and we have arbitrarily divided the scatter into those points that are above and below the line of the equation log load (kN) = − 0.33 + 0.67 (log mass (g)). This is drawn in Figure 1.

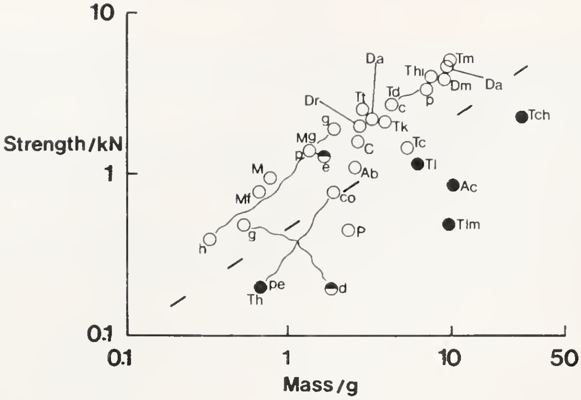

FIGURE 2. Species characterized by medians of load and of mass. Wavy lines join medians of collections made at greatly distant sites. Ab-*Acanthina brevidentata*; Ac-*Acanthina calcar;* C-*Cymia tectum;* Da-*Drupa arachnoides;* Dm-*Drupa morum;* Dr-*Drupa ricinus;* Da-*Drupella alata;* Mf-*Morula fiscella;* Mg-*Morula granulata,* e-Eilat, g-Guam, h-Hawaii, p-Palau; M-*Morula uva;* P-*Purpura patula;* Tch-*Thais chocolata;* Tc-*Thais coronata;* Td-*Thais deltoidea,* c-Curaçao, p-Panama; Th-*Thais haemastoma,* co-Costa Rica, d-Dakar, g-Ghana, pe-Peru; Thi-*Thais hippocastanum;* Tk-*Thais kiosquiformis;* Tl-*Thais lapillus;* Tlm-*Thais lamellosa;* Tm-*Thais melones;* Tt-*Thais triangularis.*

In Table I species, or populations from different localities, are listed alphabetically in three groups: all specimens above the dashed line in Figure 1; some above and some below; all specimens below the line. Of course, the more specimens of a species that are tested, the more likely it is that one or two specimens will stray above or below the line. Figure 2 attempts to demonstrate that this effect is unimportant. Each species is represented by its median value for load and median value for mass. The position of the species medians in relation to the line is seen to accord very well with the proportion of specimens falling above and below the line. From now on we shall call species all of whose shells fall above the line "strong," those that fall below "weak," and those that have specimens above and below "intermediate." This is for convenience only, and of course implies no hard and fast distinctions among species in the different categories.

All the strong species are tropical. Of the weak species, four are temperate, one is tropical, and one comes from Dakar, which might be considered as temperate. Of the intermediate species, three are tropical (*Thais haemastoma* is represented twice) and *Thais lapillus,* a temperate form, has but one shell out of 36 above the line.

The inescapable conclusion from this is that in the Thaididae the tropical shells are stronger than the temperate ones. In general the shells are about four times more resistant to crushing than temperate shells of similar mass.

We have demonstrated geographical differences in shell strength. We consider next what features of the shell may contribute to its strength or weakness. Probably the strongest shape for a shell that must grow like a snail's and which can be attacked from various directions is a thick-walled spheroid. A crude measure of the thickness of the walls is mass/volume. Table I shows that the strong species are in general thicker walled than the intermediate or weak shells. Using the species means as data points, the overall analysis of variance shows a significant difference in the means $F_{2,25} = 3.73$ $P < 0.05$. The strong shells are significantly thicker than the weak shells $F_{1,21} = 7.19$, $P < 0.05$.

There is an indication in Table I that the stronger shells are less acute. Taking species' mean acuteness from Table I as data points, the Mann–Whitney U test for the difference between the strong populations and the others lies on the border of significance ($Z = 1.91$, $P \simeq 0.05$). The difference is certainly not as marked as the difference in mass/volume. The interrelationship of these two variables is shown in Figure 3. The weaker shells are predominantly to the right and bottom of the diagram, showing that in general they are both thin-walled and acute.

The structure of the shell material also shows some relationship with shell strength, in that the three species that had rather ill-defined crossed-lamellar structure were intermediate or weak. There were two species with large amounts of calcitic accretion on the outside of the shell: *Thais coronata* and *Thais chocolata*. These species were intermediate and weak respectively.

Although the mode of fracture of crossed-lamellar structure is not well understood, Currey and Kohn (1976) have shown that the orientation of the lamellae is important in preventing cracks from traveling right through the shell. The ill-defined crossed-lamellar structure seen in three species of thaidids will be less able to interrupt crack travel than the well-defined structures. This will also be true of the calcite coating seen in two species.

DISCUSSION

In general, temperate Thaididae are considerably weaker than tropical ones, and we have also shown features of shell build and microstructure that are at least partially correlated with this. However, the selective reasons for this difference are not clear. Obviously, it should benefit any snail subject to crushing to be resistant to crushing by predators. Vermeij (1977) and Zipser and Vermeij (1978) have produced evidence that, in general, temperate shell crushers are not as strong as tropical ones. Nevertheless, the relatively weak temperate *Thais lapillus* is often killed by its local, relatively weak predators, crabs (Kitching, Muntz, and Ebling, 1966). *Thais lapillus* would therefore appear to gain a considerable selective advantage were it to evolve a stronger shell; yet it does not. Mass for mass it is far weaker than tropical shells like *Thais deltoidea* and *Drupa arachnoides*.

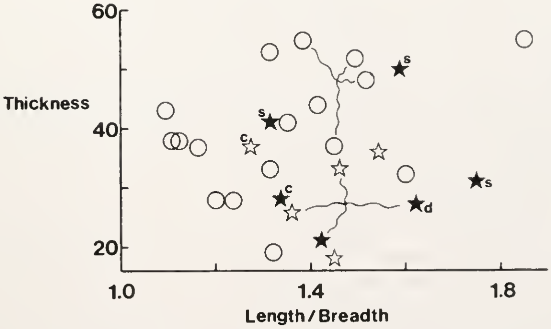

FIGURE 3. Relationship between shell thickness, spire height and strength. Ordinate = Shell thickness: $(M/V)\,10^{5}$/g mm^{-3}. Abscissa = shell acuteness: (L/B). Open circles = Strong tropical shells. Open stars = intermediate or weak tropical shells. Solid stars = intermediate or weak temperate shells. d: *Thais haemastoma* from Dakar, s: Species with ill-defined crossed-lamellar structure, c: Species with a thick layer of calcite.

These latter species are relatively thicker-walled, more spherical, and have better developed crossed-lamellar structures. Presumably these shell features are disadvantageous in other ways to a temperate-region species. One disadvantageous feature of these tropical shells is that, being relatively thicker-walled for a given volume, there is less room for the animal inside. Snails of the genus *Conus* have overcome this problem by partially dissolving the interior whorls (Kohn, Myers, and Meenakshi, 1979). Therefore, a tropical snail of given body mass has to build and carry around a more massive shell than does a relative from temperate regions. Building a relatively more massive shell may be more difficult in temperate than in tropical regions. Graus (1974) has shown that in gastropods on the Eastern seaboard of the United States, there is an almost linear relationship between mean "calcification index" (the ratio of shell mass to its internal volume) and the mean water temperature. He suggests that the main reason for this is the relative insolubility of calcium carbonate at high temperatures. This makes it easier to produce calcium carbonate structures at higher temperatures. There is other evidence that this is the case (Vermeij, 1978, pp. 16–19). However, the increased intensity of predation in the tropics must also make strong shells selectively more necessary there. Whether the indistinct shell structure seen in three of the weaker temperate species is adaptive in some way—for instance, by being metabolically cheaper or quicker to lay down—or whether it is imposed on the animal by environmental factors, is unknown.

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Summary

The crushing strength of 170 specimens of shells of 21 species of Thaididae has been measured. In general, the tropical shells are much stronger, mass for mass, than the temperate ones. This strength is achieved mainly by the shells being thicker walled in the tropics, thereby leaving less room for the animal inside.

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